University of Alberta

ADAPTIVE RESPONSES OF RAT SKELETAL MUSCLE TO ENDURANCE TRAINING AND CREATINE FEEDING

by

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ABSTRACT

Exercise training and chronic low frequency stimulation (CLFS) induce the transformation of fast-twitch fibres into slower contracting and more oxidative phenotypes in rodent skeletal muscles. The cellular conditions relating contractile activity to the expression of specific myosin heavy chain (MHC) isoforms are not yet fully understood. It is suggested that alterations in intracellular energetics may be an important physiological signal regulating muscle fibre type transitions. Previous studies have shown that muscles which undergo increases in total creatine (TCr) levels due to a creatine (Cr) loading possess an enhanced rate for high-energy phosphate shuttling. Therefore, a greater TCr content may contribute to the preservation of a faster MHC phenotype because the metabolic demand is met by a higher cellular energy status. Studies to date, however, have not examined whether Cr loading (CL) would attenuate endurance activity-induced MHC isoform transitions. Consequently, it was investigated whether chronic and acute CL combined with long-term wheel running (Run) and short-term CLFS in rats, which typically leads to fast-to-slow fibre type transitions (F-S), respectively, would 1) abolish endurance training-induced fibre type transformations, 2) alter the corresponding isometric contractile properties, 3) affect the metabolic phenotype and 4) alter proteins involved in intracellular calcium (Ca^{2+}) regulation. CL combined with either long-term Run or short-term CLFS, attenuated the activity-induced F-S in plantaris and tibialis anterior muscles, respectively. Chronic CL prevented increases in aerobic capacity while acute CL only prevented CLFS-induced

decreases in glycolytic capacity. The impact of chronic and acute CL on associated isometric contractile properties was minimal; however chronic CL decreased fatigability after 10s of stimulation. Chronic CL increased the content of the slow Ca^{2+}-ATPase, SERCA2, isoform while reducing parvalbumin protein expression. Acute CL prevented the CLFS-induced decreases in the fast Ca^{2+} ATPase isoform, SERCA1. The results of these studies are consistent with the notion that CL improves fatigue resistance and promotes changes that typically result in a more powerful muscle fibre type. There seems, however, to be a disconnect between changes in muscle phenotype and their corresponding contractile properties. This appears to be related to the adaptive changes within the proteins that regulate intracellular Ca^{2+} levels.

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TABLE OF CONTENTS

CHAPTER 1: Introduction 1

1.1 CREATINE BACKGROUND
- 1.1.1 Synthesis and degradation of creatine 1
- 1.1.2 Bioenergetics of creatine supplementation 3
- 1.1.3 Morphological manifestations with creatine supplementation 4

1.2 PERFORMANCE STUDIES (HUMAN) 4
- 1.2.1 Improvements in exercise performance 4
- 1.2.2 No improvements in exercise performance 6
- 1.2.3 Conclusions 8

1.3 FACTORS AFFECTING CREATINE LOADING 9

1.4 PROPOSED CELLULAR MECHANISMS 11

1.5 ANIMAL STUDIES 14

1.6 PURPOSES AND HYPOTHESES 16

1.7 REFERENCES FOR CHAPTER 1 32

CHAPTER 2: Reliability and sensitivity of isolated isometric function measures in rat muscles composed of different fibre types 38

2.1 INTRODUCTION 38

2.2 MATERIALS AND METHODS
- 2.2.1 Animals and surgery 40
- 2.2.2 Functional measures 41
- 2.2.3. Electrophoresis of myosin heavy chain composition 42
- 2.2.4 Statistical analyses 42

2.3 RESULTS
- 2.3.1 Isometric functional measures: force and speed parameters 43
- 2.3.2 Myosin heavy chain isoform content 44
- 2.3.3 Correlation analyses 45
- 2.3.4 Intra-animal variation 45

2.4 DISCUSSION
- 2.4.1 Isometric functional measures in relation to myosin heavy chain isoform content 60
- 2.4.2 Reliability of isometric measures 62

2.5 REFERENCES FOR CHAPTER 2 64

CHAPTER 3: Effects of long-term creatine feeding and running on isometric functional measures and myosin heavy chain content of rat skeletal muscles **67**

3.1 INTRODUCTION 67

3.2 MATERIALS AND METHODS
- 3.2.1 Animals, experimental design and surgery 69
- 3.2.2 Isometric functional measurements and muscle sampling 71
- 3.2.3 High-energy phosphate and total creatine measures 72
- 3.2.4 Electrophoretic analyses of myosin heavy chain content 73
- 3.2.5 Statistical analyses 73

3.3 RESULTS
- 3.3.1 Food intake, solution intake, creatine consumption and distance ran 74
- 3.3.2 Body, muscle and muscle-to-body mass ratio 74
- 3.3.3 Isometric functional measures of force, speed and fatigability 75
- 3.3.4 High-energy phosphates and total creatine levels 76
- 3.3.5 Myosin heavy chain isoform content 76

3.4 DISCUSSION
- 3.4.1 Anthropometrical parameters, activity level and diet 93
- 3.4.2 Creatine and high-energy phosphate levels 94
- 3.4.3 Contractile properties and myosin heavy chain isoform content 95
- 3.4.4 Influences of the intramuscular phosphorylation potential on myosin heavy chain isoform expression 96

3.5 REFERENCES FOR CHAPTER 3 98

CHAPTER 4: Adaptive responses to creatine loading and voluntary exercise in rat skeletal muscle **103**

4.1 INTRODUCTION 103

4.2 MATERIALS AND METHODS
- 4.2.1 Animals, experimental design and muscle sampling 105
- 4.2.2 Immunohistochemical staining and analyses of myofibres with antibodies against myosin heavy chain isoforms 106
- 4.2.3 Western blot analyses of parvalbumin and calcium ATPases 107
- 4.2.4 Metabolic enzyme analyses 109
- 4.2.5 Statistical analyses 110

4.3 RESULTS
4.3.1 Fibre type transitions 110
4.3.2 Cross-sectional areas of fibres 111
4.3.3 Parvalbumin and calcium ATPase content 111
4.3.4 Reference enzyme activities 112

4.4 DISCUSSION
4.4.1 Fibre type transitions 131
4.4.2 Calcium regulatory proteins 133
4.4.3 Metabolic profile 135
4.4.4 Conclusions 135

4.5 REFERENCES FOR CHAPTER 4 137

CHAPTER 5: Chronic low frequency stimulation and creatine loading affects structural and metabolic properties in rat skeletal muscle **143**

5.1 INTRODUCTION 143

5.2 MATERIALS AND METHODS
5.2.1 Animals and experimental design 145
5.2.2 Surgery and chronic low frequency stimulation 146
5.2.3 Isometric functional measurements and muscle sampling 146
5.2.4 Total creatine levels 147
5.2.5 Electrophoresis, immunohistochemistry and real-time reverse transcriptase polymerase chain reaction of myosin heavy chain isoform content 148
5.2.6 Western blot analyses of parvalbumin and calcium ATPases 148
5.2.7 Metabolic enzyme analyses 149
5.2.8 Statistical analyses 149

5.3 RESULTS
5.3.1 Body, muscle and muscle-to-body mass ratio 150
5.3.2 Solution intake, creatine consumption and total creatine levels 150
5.3.3 Isolated isometric measures of force and contraction speed 151
5.3.4 Myosin heavy chain isoform and mRNA content 151
5.3.5 Parvalbumin and calcium ATPases content 152
5.3.6 Oxidative and glycolytic enzyme content 153

5.4 DISCUSSION
5.4.1 MHC-based fibre type transitions and functional measures 181
5.4.2 Parvalbumin content 184
5.4.3 Calcium ATPase content 185
5.4.4 Metabolic profile 186
5.4.5 Conclusions 188

5.5 REFERENCES FOR CHAPTER 5 189

CHAPTER 6: GENERAL CONCLUSIONS 196

6.1 SUMMARY 196

6.2 CREATINE INFLUENCES CELLULAR ENERGETIC STATUS 198

6.3 MYOSIN HEAVY CHAIN-BASED FIBRE TYPE ADAPTATIONS RUNNING AFTER AND CHRONIC LOW FREQUENCY STIMULATION COMBINED WITH CREATINE 200

6.4 DISCONNECTION BETWEEN MHC-ISOFORM PROTEIN EXPRESSION AND FUNCTIONAL PROPERTIES 207

6.5 DISCONNECT BETWEEN MHC AND CALCIUM REGULATORY PROTEINS 208

6.6 CLINICAL RELEVANCE 209

6.7 REFERENCES FOR CHAPTER 6 212

LIST OF TABLES

CHAPTER 2: Reliability and sensitivity of isolated isometric function measures in rat muscles composed of different fibre types

Table 2-1: Correlation coefficients derived from multiple regression analyses of myosin heavy chain isoforms and isometric functional measurements. 55

CHAPTER 3: Effects of long-term creatine feeding and running on isometric functional measures and myosin heavy chain content of rat skeletal muscles

Table 3-1: Muscle mass and muscle-to-body mass ratio of rats after creatine feeding and exercise training. 78

Table 3-2: Contractile properties of soleus, extensor digitorum longus, medial gastrocnemius and plantaris muscles after creatine feeding and exercise training. 79

Table 3-3: Isometric force production of soleus, extensor digitorum longus, medial gastrocnemius and plantaris muscles after creatine feeding and exercise training. 80

Table 3-4 Concentrations of high-energy phosphates in medial gastrocnemius and plantaris muscles after creatine feeding and exercise training. 81

Table 3-5: Myosin heavy chain isoform composition of rat soleus, extensor digitorum longus and medial gastrocnemius muscles. 82

CHAPTER 5: Chronic low frequency stimulation and creatine loading affects structural and metabolic properties in rat skeletal muscle

Table 5-1: Rat specific real-time polymerase chain reaction primers and probes. 154

Table 5-2: Muscle mass and tibialis anterior-to-body mass ratio in control and stimulated/sham hindlimbs after creatine feeding and chronic low frequency stimulation treatment. 155

Table 5-3: Wet-to-dry ratio of tibialis anterior in control and stimulated/sham hindlimbs after creatine feeding and chronic low frequency stimulation treatment. 156

LIST OF FIGURES

CHAPTER 1: Introduction

Figure 1-1:	The structural formula of creatine.	21
Figure 1-2:	Biosynthesis of creatine.	23
Figure 1-3:	Creatine transport.	25
Figure 1-4:	Creatine kinase reaction and creatinine formation.	27
Figure 1-5:	Phosphocreatine circuit shuttling system.	29
Figure 1-6:	Schematic of the chronic low frequency stimulation model.	31

CHAPTER 2: Reliability and sensitivity of isolated isometric function measures in rat muscles composed of different fibre types

Figure 2-1:	Isolated isometric speed parameters of soleus, extensor digitorum longus and medial gastrocnemius muscles.	48
Figure2-2:	Isolated isometric functional measures of soleus, extensor digitorum longus and medial gastrocnemius muscles.	50
Figure 2-3:	Electrophoretic method used to quantify myosin heavy chain isoform composition in soleus, extensor digitorum longus, medial gastrocnemius and plantaris muscles.	52
Figure 2-4:	Myosin heavy chain isoform composition and phenotypic factor of soleus, extensor digitorum longus and medial gastrocnemius muscles.	54
Figure 2-5:	Comparison of isolated isometric functional measures between soleus, extensor digitorum longus and medial gastrocnemius muscles of the right and left hindlimbs.	57
Figure 2-6:	Absolute reliability scores of isometric functional measures for soleus, extensor digitorum longus and medial gastrocnemius muscles.	59

CHAPTER 3: Effects of long-term creatine feeding and running on isometric functional measures and myosin heavy chain content of rat skeletal muscles

Figure 3-1: Daily food intake and daily volume of solution consumed by all treatment groups. 84

Figure 3-2: Averaged fatigue index calculated after 10s and after 30s of stimulation in the soleus, extensor digitorum longus, medial gastrocnemius and plantaris muscles. 86

Figure 3-3: Total creatine content in the plantaris muscle after 13-week study. 88

Figure 3-4: Electrophoretic method used to separate and quantify myosin heavy chain isoforms in the soleus, extensor digitorum longus, medial gastrocnemius and plantaris muscles. 90

Figure 3-5: Myosin heavy chain isoform composition of plantaris muscles. 92

CHAPTER 4: Adaptive responses to creatine loading and voluntary exercise in rat skeletal muscle

Figure 4-1: Representative photomicrographs of immunohistochemistry of the plantaris muscles. 114

Figure 4-2: The percentage of fibres expressing a particular myosin heavy chain in the plantaris muscles. 116

Figure 4-3: The percentage of pure and hybrid fibre types in the plantaris muscles. 118

Figure 4-4: Cross-sectional area of fibre types in the plantaris muscles. 120

Figure 4-5: Myofibre size distribution (Type IIA, IID(X) and IIB) of the plantaris muscles. 122

Figure 4-6: A representative immunoblot and densitometric evaluation of parvalbumin in the plantaris muscles. 124

Figure 4-7: A representative immunoblot and densitometric evaluation of the fast Ca^{2+} ATPase isoform, SERCA1, in the plantaris muscles. 126

Figure 4-8: A representative immunoblot and densitometric evaluation of the slow Ca^{2+} ATPase isoform, SERCA2, in the plantaris muscles. 128

Figure 4-9: Maximum activities of citrate synthase, 3-hydroxyacyl-CoA dehydrogenase, glyceraldehyde phosphate dehydrogenase and phosphofructokinase in the plantaris muscles. 130

CHAPTER 5: Chronic low frequency stimulation and creatine loading affects structural and metabolic properties in rat skeletal muscle

Figure 5-1: Changes in body mass during 20 days of creatine loading. Chronic low frequency stimulation was applied during the last 10 days of creatine loading. 158

Figure 5-2: Daily solution intake during 20 days of creatine loading. 160

Figure 5-3: Daily creatine consumption in the Cre-Stim and Cre-Sham groups during 20 days of creatine loading. 162

Figure 5-4: Total creatine within the tibialis anterior muscles of creatine and dextrose (Control) fed groups. 164

Figure 5-5: Isometric functional measures of the tibialis anterior muscles. 166

Figure 5-6: Electrophoretic method used to separate and quantify myosin heavy chain isoform composition of tibialis anterior muscle. 168

Figure 5-7: Myosin heavy chain isoform composition of tibialis anterior muscles. 170

Figure 5-8: Myosin heavy chain mRNA content (ΔCt) assessed via real-time polymerase chain reaction in the tibialis anterior muscles. 172

Figure 5-9: A representative immunoblot and densitometric evaluation of parvalbumin in the tibialis anterior muscles. 174

Figure 5-10: A representative immunoblot and densitometric evaluation of the fast Ca^{2+} ATPase isoform, SERCA1, in the tibialis anterior muscles. 176

Figure 5-11: A representative immunoblot and densitometric evaluation of the slow Ca^{2+} ATPase isoform, SERCA2, in the tibialis anterior muscles. 178

Figure 5-12: Maximum activities of citrate synthase, 3-hydroxyacyl-CoA dehydrogenase, glyceraldehyde phosphate dehydrogenase and creatine kinase in the tibialis anterior muscles. 180

CHAPTER 6: General conclusions

Figure 6-1 Diagram of the potential influences of Cr feeding on muscle fibre type phenotype. 206

LIST OF ABBREVIATIONS

ΔCt	change in threshold value
AICAR	5-aminoimidazole-4-carboxamide-1-ß-D-ribofuranoside
β-GPA	β-guanidinopropionic acid
½ FT	half-fall time
½ RT	half-rise time
ANOVA	analysis of variance
ADP	adenosine diphospate
AGAT	L-arginine: glycine amidinotransferase
ATP	adenosine triphospate
Ca^{2+}	calcium
CHO	carbohydrate
CK	creatine kinase
CLFS	chronic low frequency stimulation
Cr	creatine
Crn	creatinine
CS	citrate synthase
[ ]	concentration
CSA	cross-sectional area
DNA	deoxyribonucleic acid
EDL	extensor digitorium longus
FI	fatigue index
G	gram
GAMT	S-adenosylmethionine: guanidinoacetate methyltransferase
GAPDH	glyceraldehydes phosphate dehydrogenase
GI	gastro-intestinal
HADH	3-hydroxyacyl-CoA dehydrogenase
hr(s)	hour(s)
IPP	intracellular phosphorylation potential
Km	kilometre
L_0	optimal resting length
M	metre
MG	medial gastrocnemius
min(s)	minute(s)
MHC	myosin heavy chain
MLC	myosin light chain
mRNA	messenger ribonucleic acid
n	sample size
N_2	nitrogen
Na^+	sodium
P_i	inorganic phosphate
PCr	phosphocreatine
PFK	phosphofructokinase
PL	plantaris
R	reliability

LIST OF ABBREVIATIONS

s	second
SEM	standard error of mean
SERCA1	fast sarco/endoplasmic reticulum calcium ATPase isoform
SERCA2	slow sarco/endoplasmic reticulum calcium ATPase isoform
SOL	soleus
SR	sarcoplasmic reticulum
TA	tibialis anterior
TCr	total creatine
TET	tetanus force
TTP	time-to-peak twitch
TW	twitch force

CHAPTER 1: Introduction

1.1 CREATINE BACKGROUND

1.1.1 *Synthesis and degradation of creatine*

Creatine (Cr) supplementation studies have shown that ingesting this natural compound leads to increased intramuscular total Cr within a few days (Harris et al. 1992), and results in enhanced muscular power generation and endurance (Balsom et al. 1993). Cr is a nitrogenous molecule (Figure 1-1) that exists naturally in muscles of humans and mammals. Most meat diets contain on average 1 g of Cr daily, but an average person requires 2 g to replace Cr catabolism as a result of body metabolism. The difference between these amounts is derived from endogenous synthesis of Cr, which occurs in the liver, kidney and in the testes to some extent (Hunter 1922). Cr is synthesized from glycine and arginine in a two-step process (Figure 1-2). L-arginine: glycine amidinotransferase (AGAT) catalyzes the transfer of the amidino group of arginine to glycine to form L-ornithine and guanidinoacetate. The latter compound by the action of S-adenosylmethionine: guanidinoacetate methyltransferase (GAMT) is methylated to produce Cr (Bloch and Schoenheimer 1940).

The highest levels of Cr and PCr are observed in skeletal muscle, heart, spermatozoa and photoreceptors in the retina. In human skeletal muscle, [Cr] and [PCr] correlate with glycolytic capacity, with type IIB fibres containing 32mM PCr and 7mM Cr, while type I fibres contain 16mM PCr and 7mM Cr at rest (Kushmerick et al. 1992). Cr requires transport of intermediates between the liver and kidneys. Cr is taken up by the muscles against a concentration gradient by a sodium-dependent saturable active transport process. Cr uptake via Cr transporters is driven by electrochemical potential differences of extracellular versus intracellular sodium concentrations (Figure 1-3). Two Na^+ ions (~30KJ)

provide the energy to move Cr up its concentration gradient. Creatinine (Crn) is the only end product of Cr degradation (irreversible reaction, Figure 1-4). Production of Crn is followed by its entry into the circulation via simple diffusion and is then excreted in the urine after filtration in the kidneys. Since skeletal muscle is the major store of Cr pool, it is also the main production site of Crn. Cr (1.1%/day) and PCr (2.6%/day) non-enzymatically convert to Crn, giving an overall conversion rate for the total Cr pool (Cr + PCr) of approximately 1.7%/day (Walker 1979). The biodegradation of Cr into Crn is dependent upon pH and temperature (Ennor and Morrison 1998). Creatine phosphate (PCr) is the form in which Cr is entrapped in the muscle because it is unable to pass through plasma membrane. PCr is used to maintain ATP regeneration in the following manner:

$$Cr\text{-}P + ADP + H^+ \Leftrightarrow Cr + ATP \text{ (via CK activity, Figures 1-4 and 1-5)}$$

The ATP regeneration capacity of creatine kinase (CK) is very high and exceeds both ATP utilization as well as ATP replenishment by oxidative phosphorylation and glycolysis (McGilvery 1975). In addition, CK has a low-threshold ADP sensor (K_m of MM-CK for ADP is 10-35 μM), which prevents the accumulation of ADP (Matthews et al. 1982).

The CK/PCr (kinase/phosphocreatine) system is considered to serve many functions.

1. CK/PCR system is a temporal and spatial energy buffer (Bessman and Geiger 1981). PCr acts as an "energy carrier" connecting sites of production and utilization through compartmentalized CK isozymes.
2. It prevents a rise in intracellular [free ADP]. Indirectly, avoiding an inactivation of ATPases and a net loss of adenine nucleotides (Iyengar et al. 1982).
3. A release of P_i occurs in the CK/PCr system, which results from the hydrolysis of PCr. P_i is needed to activate certain enzymes in

glycogenolysis and glycolysis, and thus indirectly affects the activation of these metabolic processes.

4. It may regulate local ATP/ADP ratios at sites where CK is coupled to ATP-consuming enzymes. By maintaining a high ATP/ADP ratio in the vicinity of ATPase, CK increases the efficiency of ATP hydrolysis.

PCr stores are finite and can fall dramatically during the first few seconds of maximal intensity exercise. Söderlund and Hultman (1991) observed drops in PCr and ATP levels with electrical stimulation to the quadriceps femoris of healthy subjects. Single fibres from biopsies of the vastus lateralis revealed that 52 contractions lead to 40% and 47% reduction in ATP levels and 93% and 94% drops in PCr content in type I and type II fibres, respectively. After 15min of recovery, ATP levels in type I fibres but not type II fibres were completely regained; whereas, PCr content returned to pre-exercise values after 5min of recovery in both fibre types. The findings of Harris et al. (1976) showed that the resynthesis of PCr in humans is bi-phasic in nature with a fast and slow component with half-times of 20-22s and 170s, respectively. Nutritional manipulations that lead to an increase in PCr in muscle should result in greater work capacities and longer maintenance of high-intensity exercise due to a delay in fatigue onset. Muscular fatigue is believed to be a result of ATP concentrations falling to approximately 25-30% (Hultman et al. 1991) among other factors. Matching ATP regeneration to ADP hydrolysis via Cr utilization might delay increase recovery of fatigue resistance. Greenhaff et al. (1994) were the first to show that an increase in muscle Cr concentration as a result of dietary Cr supplementation can accelerate the rate of muscle PCr resynthesis during recovery from exercise.

1.1.2 *Bioenergetics of creatine supplementation*

Raising blood Cr concentrations above normal levels by ingesting Cr could lead to morphological changes in muscle structure. Several dosages were

tested, and five grams (5g) of Cr resulted in optimal dosage causing the greatest rise in plasma Cr. Rise in total Cr (TCr) (20%) was due to increases in both PCr and Cr (Harris et al. 1992). The increase in TCr appeared to be less dependent upon duration of supplementation and daily dose rate than upon initial TCr content. Tesch et al. (1989) reported that PCr levels are resynthesized 67% and 50% of the value at rest in type I and type II fibres after 1min of recovery from intense knee extensor contractions; thus, suggesting that slow twitch fibres have a greater capacity to resynthesize PCr. Theoretically, this increased ATP turnover rate should improve performance.

1.1.3 *Morphological manifestations with creatine supplementation*

Cr has proven to augment total body mass, fat free mass and muscle strength (Greenhaff et al. 1994; Dawson et al. 1995; Earnest et al. 1995). Compared with placebo, cross-sectional areas of Cr loaded skeletal muscle revealed greater hypertrophy of type I, IIA and IIB muscle fibres (Volek et al.1999). At present, no measurements have been made on myofibrillar protein content, MHC mRNA and protein expression. Thus, the physiological mechanisms through which Cr affects fibre isoforms are undefined. Proposed mechanisms responsible for the reported increases in body mass, strength and muscular endurance will be discussed later in this section.

1.2 PERFORMANCE STUDIES (HUMAN)

1.2.1 *Improvements in exercise performance*

Some studies have shown that Cr enhances physical performance. The maintenance of force production during evoked muscle contractions is apparently augmented.

i) Repeated-bouts of high-intensity exercise:

Balsom et al. (1995) reported improvements in power output in subjects performing repeated bouts (5 sets of 6s with 30s recovery) of high-intensity exercise, on a cycle ergometer after 6 days (20g Cr /day) of Cr supplementation. As a result of the Cr supplementation, TCr was increased at rest, and after the 5th exercise bout PCr concentration was also higher. Another experimental protocol of Greenhaff et al. (1993) depicted increased total peak torque generation during the 2nd and 3rd bouts of unilateral isokinetic knee extension after Cr ingestion (4 x 5 g of Cr + 1 g of glucose/day). One bout represented 30 maximal contractions (this quantity was chosen because it shows a marked PCr degradation in both type I and II fibres in quadriceps (Tesch 1989)). Subjects undertook 5 bouts of 30 maximal contractions, interspersed by a minute recovery before and after 5 days of Cr or placebo. A study by Birch et al. (1994) revealed that the rate of decline of power output that occurs during repeated bouts of supramaximal intensity cycle exercise was significantly less after Cr supplementation (4 x 5 g/day for 5 days). Cr ingestion increased peak power output by 8%, mean power output by 6% and total work output. Overall, Cr was able to maintain the required rate of ATP resynthesis needed for repeated exercise periods. Increased performance may be due to Cr achieving a higher muscle ATP turnover rate during contraction. Trump et al. (1996) examined the importance of PCr degradation in maintaining power output during maximal intermittent cycling in men during 3 bouts of isokinetic cycling (30s, 100 revs/min) with 4 min of rest between bouts. The results suggested that PCr contributed approximately 15% of the total ATP provision during the third 30-s bout of maximal isokinetic cycling and that most of the ATP was provided during the initial 15s.

ii) Resistance exercise:

Earnest et al. (1995) used 10 weightlifters and a protocol which involved 3 consecutive 30s Wingate bike test, 1 repetition maximum (RM) bench press and a 70% 1 RM on the bench press until fatigue. Results showed that total anaerobic work for Wingate tests were higher during the Cr trials, and total lifting volume was also higher in the Cr group (20g/d), which performed 26% more lifting repetitions after an acute 5 day load. In another Cr investigation, twenty-two studies were reviewed and it was reported that the average increase in muscle strength (1, 3, or 10 RM) and weightlifting performance (maximal repetitions at a given percent of maximal strength) following Cr supplementation plus resistance training were 8% and 14% greater, respectively, than the average increase in muscle strength following placebo ingestion during resistance training. Overall, Cr supplementation during resistance training is more effective at increasing muscle strength and weightlifting performance than resistance training alone, although the response is highly variable (Rawson and Volek 2003).

1.2.2 *No improvements in exercise performance*

The findings in exercise performance enhancement with Cr supplementation are equivocal. There are numerous studies that report no significant increases in muscular power, strength or PCr resynthesis following either a short term (5 day, 20g/d) or long-term ingestion period.

i) Endurance exercise:

A study by Balsom et al. (1993) where subjects were given 20g/day of Cr for 6 days showed no improvement in performance time in a 6 km cross-country run. Supplementation actually increased the volunteers running time, likely due to the increases in body mass. Since most Cr supplementation studies report an

increase in total body weight, even with a typical short-term 5-day load, this extra body mass may hinder performance in longer duration activities, where strength to mass ratio may be critical (i.e. marathon). Syrotuik and colleagues (2001) showed that subjects ingesting Cr for 6 weeks (5 day load dose (0.3 g/kg/d) followed by 5 weeks of maintenance dose (0.03 g/kg/d) in a combined high intensity rowing and strength program did not perform more repetitions per set of strength training nor produce or maintain higher power outputs during repeated rowing sessions compared to their placebo counterparts. Overall, Cr supplementation did not increase performance or training volume.

ii) Short-duration exercises (maximal intensity):

Cooke et al. (1995) showed no improvements in performance. They measured power output during 15 seconds of maximal cycling sprints against a constant load between placebo and Cr fed subjects (5g of Cr + 1g of glucose for 5 days). This study also reported no significant differences between groups for peak power, time to peak power or total work. Similarly, Odland et al. (1997) showed no differences in mean peak 10s power output, mean 30s power output, percent fatigue or post-exercise lactate accumulation between control and Cr fed (20g/day for 3 days) groups during 30s maximal cycling sprints. Another study showed that 5 days of Cr supplementation (4 x 5 g/day) did not improve multiple sprint measures of fastest time (15 x 30m repeated at 35s intervals), mean time, fatigue, or post-test blood lactate concentration in men (Glaiser et al. 2006). It was concluded that despite widespread use of Cr as an ergogenic aid, the findings suggested that Cr supplementation conveyed no benefit to multiple sprint running performance.

iii) Resistance exercise:

A study by Syrotuik et al. (2000) revealed no significant differences in absolute strength (1RM), total lifting volume and strength per mass ratio between

experimental groups (placebo, acute 5-day load (0.3g/kg/d) with and without a 32 day maintenance dose of 0.03g/kg/d) for bench and incline leg press. These findings suggest that Cr supplementation with resistance training does not result in an absolute or relative training advantage. Similarly, a study by Ferguson and Syrotuik (2006) showed that 10 wks of Cr supplementation at a dose of 0.3 g/kg/d and 0.03 g/kg/d body mass for the initial 7 days and subsequent 9 weeks, respectively, coupled with resistance training in women did not significantly increase 1 repetition maximum (1RM) bench press and incline leg press. There was a main effect for training, but there was no significant difference in the total number of repetitions completed after 5 sets of multiple repetitions to exhaustion at 70% of 1RM for bench press and incline leg press or in the performance of a greater training volume. A study by Stevenson and Dudley (2001) also revealed no significant changes in unilateral strength or multi-set resistance exercise performance for knee extensions. The Cr group (20g/d for 7 days) did not experience increases in voluntary isokinetic torque or in rate of torque development when performing a 1RM or a 5-set performance for unilateral, dynamic knee extension. Overall, these studies suggest that Cr loading does not augment muscular strength.

1.2.3 *Conclusions*

Although the results may be somewhat equivocal with the effects of Cr supplementation and any ergogenic boost provided by either short or long term ingestion, the majority of the literature suggests a performance enhancement in a variety of short duration, high power activities. This effect appears to be more pronounced when the activity relies predominately on the high-energy phosphagen system of ATP-PC and is repeated, with limited recovery time between bouts or repetitions of exercise (Terjung et al. 2000).

1.3 FACTORS AFFECTING CREATINE LOADING

The inconsistencies found in the relevant literature about Cr as an ergogenic aid are not uncommon when evaluating nutritional supplements. The differences in data may be a result from varying study designs, and duration and dosage of Cr supplementation. Subject variability is also a powerful determinant of the effects of Cr supplementation. The subject's age, training status (blood flow), hydration status, initial levels of TCr, fibre type, diet (high in carbohydrate), motivation, tolerance of discomfort, perception of fatigue, familiarity with exhaustive exercise will affect the results, and hence the efficiency of Cr to enhance performance. Age has proven to be an important factor when considering Cr supplementation. Differential effects of Cr depletion on MHC transitions appear to be age dependent in animal models, where significant depletion of Cr occurs in very young animals (weaning age). This imposition is greater in growing rats and less pronounced in older animals (Adams and Baldwin 1995). In addition, muscles composed of different fibre types may display varying capacities for Cr uptake and retention. Op 't Eijnde (2001) showed that short-term (5 days) high dose (5g/kg body wt/day) Cr feeding enhanced TCr content in soleus (SOL) (+ 20%) but not in red gastrocnemius and white gastrocnemius muscles. Therefore, Cr supplementation was concluded to be markedly greater in slow oxidative than in fast glycolytic muscle fibres.

A study by Greenhaff et al. (1994) found that oral Cr ingestion (20g for 5 days) increased Cr uptake in those individuals who had a total resting muscle Cr concentration of close to 120 mmol/kg•dm before ingestion. The mean total Cr concentration of human skeletal muscle in this study was 124.4 ± 11.2 mmol/kg dry matter (81 biopsies), and followed a normal distribution of 90 to 170 mmol/kg•dm. Levels of intramuscular TCr prior to dosage administration have become an important factor in determining the effects of Cr supplementation in humans.

Large inter-subject variation in muscle Cr retention from Cr supplementation may be due to differences in diet. It has been suggested that diets, which include large intakes of carbohydrates (CHO), may enhance the effects of Cr intake. Green et al. (1996a and 1996b) demonstrated that CHO loading resulted in significantly higher increases in skeletal muscle Cr content when compared to Cr supplementation alone. The findings demonstrated that CHO ingestion substantially augmented muscle Cr accumulation during Cr feeding and appears to be insulin mediated. Cr harnesses the potential energy of Na^+ wanting to enter the muscle cell. Thus, any agent that increases the extrusion of Na^+ should increase the transport of Cr into the muscle cell. Physiological agents that increase Na^+ efflux include insulin and epinephrine. Since two Na^+ ions need to accompany each Cr molecule being transported inward, doubling the driving force for Na^+ inwardly will increase Cr transport by 50% (refer to Figure 1-3). In general, high CHO diets and Cr supplementation exert a synergistic effect on Cr uptake by affecting Cr transport into the muscle cell.

The exercise protocol developed and utilized in the study designs may also affect the results obtained. The type, duration, intensity, volume of exercise, and recovery period (rest between sets) are important factors to consider when evaluating the effects of Cr supplementation. Cr supplementation exerts its greatest effects on activities which employ high-power output efforts maintained for only a short period of time (few seconds), and separated by a short rest period (20-60 s). A study by Syrotuik and Bell (2004) described the physiological profile of responders vs. non-responders when fed Cr (0.3 g/kg/d) for 5 days. It was suggested a person-by-treatment interaction to acute Cr feeding occurred, with responders possessing lowest initial levels of Cr, greatest percentage of type II fibres, and greatest preload muscle fibre cross-sectional area and fat-free mass. This study may aid in partially explaining the equivocal data found in the Cr literature.

1.4 PROPOSED CELLULAR MECHANISMS

It has been suggested that fibre hypertrophy and increased protein synthesis, as a result of Cr intake, are responsible for the reported athletic performance improvements seen mostly when Cr is combined with resistance training. The early works of Ingwall reported that Cr supplementation enhanced protein synthesis in skeletal and cardiac muscle (Ingwall et al. 1972 and 1975; Ingwall, 1976). The results suggest that Cr stimulates muscle-specific protein synthesis; namely, actin, MHC and the cytosolic isoform of CK (MM-CK) in cultures of differentiating muscle cells. Interestingly, however, Cr did not alter the synthesis rates of soluble metabolic enzymes. These results indicate that Cr may function as a transcriptional or translational factor, or may alter charged tRNAs or amino acid pools, which may be specific for myofibrillar protein synthesis.

Cr administration, in combination with increased functional loading, results in increased satellite cell mitotic activity. This phenomenon could possibly explain the muscle growth and compensatory hypertrophy seen with Cr intake. A study by Dangott, Schultz and Mozdiak (2000) showed that the index of satellite cell mitotic activity was significantly higher in myofibres after prolonged Cr feeding. Moreover, myofibres were larger in both mass and diameter measurements. Oral Cr supplementation may increase Cr levels within myofibres, which in turn increases osmotic pressure of myofibres. This increased pressure may cause an increase in muscle size and indirectly signal satellite cells to proliferate and fuse with growing myofibres. The specific mechanism by which muscle hypertrophy occurs is unknown, but it is most likely that Cr enhances muscle hypertrophy/myonuclear accretion through a myofibre mechanism because satellite cell mitotic activity is directed by the myofibres themselves. Olsen et al. (2006) investigated the influence of Cr supplementation on satellite cell frequency and number of myonuclei in human skeletal muscle during 16 weeks of heavy-resistance training. Muscle biopsies showed greater

increases in muscle mean fibre area, the proportion of satellite cells (SC) and number of myonuclei per fibre in the Cr fed group. The authors concluded that Cr supplementation in combination with strength training amplifies the training-induced increase in SC number and myonuclei concentration in skeletal muscle fibres, thereby allowing an enhanced muscle fibre growth in response to strength training.

Hormones can affect cell volume by altering amino acid transport across the plasma membrane and intracellular metabolism. Cell volume changes can affect alterations in cellular function. Cell swelling, for example, triggers an anabolic pattern of cellular metabolism; namely, inhibits glycogenolysis, glycolysis and proteolysis and stimulates glycogen synthesis, amino acid mobilization and utilization (Haussinger and Lang, 1994). Retention of Cr coincides with a reduction in urine production on the first 3 days of a Cr loading period (Hultman et al. 1996). This retention of water is thought to be related to the osmotic load caused by Cr retention and to account for the rapid onset of weight gains (Ziegenfuss et al. 1998). The mechanisms that link cell volume and cellular function are unclear, as are the signals initiated by hormone receptor activation; however, intact microtubular structures are required. Disruption of microtubules by colchicines abolishes the anti-proteolytic actions of insulin-induced cell swelling (Haussinger et al. 1994). In addition, amino acid, insulin-induced cell swelling leads to alkalinization of intracellular vesicular compartments (lysosomes and endocytotic compartments). Since proteolysis is dependent on an acidic pH, a link has been made between cellular hydration and proteolytic activity. If Cr increases cellular hydration, it may increase protein synthesis and increase fat-free mass. Regulation of protein turnover by the cellular hydration state as determined by the activity of ion and substrate transport systems in the plasma membrane has been shown in liver cells, but it may also occur in skeletal muscle (Haussinger et al. 1990).

Another proposed mechanism involves the permissive actions of ATP. Extracellular ATP is mitogenic in vascular smooth muscle cells and stimulates several events that are needed for cell proliferation; namely, DNA synthesis, protein synthesis and increase in cell number. The signal transduction is mediated by G-proteins, phospholipase C*B* and D, diacyl-glycerol, protein kinase C Raf-1, MEK and MAPK (Erlinge 1998). The mitogenic effects have been demonstrated in rat, bovine, and humans. The mitogenic actions of ATP have been proven in smooth muscle; however it is unknown whether ATP has the same actions in skeletal muscle. Such mechanisms may be responsible for the reported increases in protein synthesis associated with Cr supplementation.

Since Cr supplementation has proven to increase muscle mass and TCr pools, it's reasonable to investigate the consequences of Cr depletion in order to emphasize the effects of Cr. β -guanidinoproprionate (β -GPA) competitively inhibits Cr transporter activity and reduces Cr import through the sarcolemma. Therefore, PCr, Cr and ATP levels decrease. β-GPA is used as an antagonist (competitive inhibition) in animal studies in order to study muscle function, energetics, fibre type changes and protein expression. β-GPA leads to decreases in Cr, PCr and ATP (Ren et al. 1995). In addition, the enzymatic assays of Foley et al. (1994) discovered that β-GPA decreased ATP by 40%, PCr by 87% and TCr by 77%. These results exhibit a reduction in energy-rich phosphates and a reduced phosphorylation potential, which in turn appear to affect isoform expression in the fast-twitch skeletal muscles of rats. Namely, β-GPA caused an increase in slow isomyosin and a decrease in fast isomyosins. Adaptations to chronic depletion of Cr include reduced fibre diameter; probably to decrease diffusion distance for energy metabolites; increases in GLUT 4 transporters in order to increase availability of energy sources (ATP) for proper function, and decreased glycolytic potential. High energy stress appears to induce compensatory measures, which serve to maintain the supply of ATP and to limit the maximum rate of ATP consumption. However, the relationship between the energy potential and the MHC-based phenotype remains unclear.

1.5 ANIMAL STUDIES

In comparison to human performance studies, there have been fewer investigations that assessed the effects of Cr on muscle structure and function using animal models. Until now, previous animal research had not considered the potential impact of Cr on the patterns of MHC isoform expression and related fibre type distribution after Cr supplementation or the corresponding effects on contractile properties and energy metabolism.

The studies in this dissertation are the first investigations to examine the long-term and short-term effects of Cr feeding combined with voluntary running and chronic low frequency stimulation (CLFS) on rat skeletal muscle structure and function. Most of the studies completed to date have only examined the acute effects of Cr feeding, but have not attempted to study the consequences of long-term Cr feeding. A rat model has the advantages of minimizing the psychological constraints associated with using human subjects, where measures of muscle power output and fatigue resistance may be underestimated. These studies employ very rigorous analytical measures, and an experimental approach designed to maximize the effects of Cr on muscle structure and function. The effects of Cr loading are examined combined with exercise and glucose feeding during the most rapid growth phase of the Sprague-Dawley rat, thereby allowing for maximal Cr uptake. The analytical outcomes allow for the direct correlation of functional improvements in twitch characteristics, strength and fatigue resistance with quantitative and qualitative changes to skeletal muscle fibre morphology and biochemistry.

Throughout these studies, the following assumptions are made:

1) The running groups (see Chapter 3) ingest more food and water than the sedentary groups. Therefore, the sedentary group is expected to consume less Cr. However, matching and randomly assigning the animals has diminished variation.

2) All rats respond to the CLFS to the same extent (see Chapter 5).

3) The degree to which the results translate to humans is not completely known. However, as previously noted some human studies have shown increased performance (strength and endurance), increased muscle mass and hypertrophy with Cr supplementation. A "non-response" effect is present in 30% of human subjects who undergo a loading protocol and do not respond to the augmented [TCr] (Greenhaff, 1997). It follows, that the observed effects may also exist within a rat model of Cr administration.

The results from these studies provide a basic understanding of how chronic and acute Cr feeding influences muscle fibre type phenotype and whole muscle function. In addition, it determines the role of Cr in the regulation of skeletal muscle synthesis and hypertrophy via examination of the fibres' cross-sectional area. The results from these sets of experiments have implications for understanding some of the factors which regulate fibre type transitions. In addition, it will lead to a better understanding of the potential for Cr feeding to positively influence skeletal muscle structure and function in health and in diseased conditions such as muscular dystrophies where Cr loss is substantial.

1.6 PURPOSES AND HYPOTHESES

The cellular conditions relating contractile activity to the expression of specific myosin heavy chain (MHC) isoforms are not yet completely identified. It is suggested that changes in intracellular energetics represent an important physiological signal regulating muscle fibre type transitions. Previous studies (Ingwall 1976; Bessman et al. 1987) have shown that muscles which undergo increases in total creatine (TCr) levels due to a Cr feeding possess an enhanced rate for high-energy phosphate shuttling. It follows that a greater TCr content may contribute to the maintenance of a faster MHC phenotypic profile because the metabolic demand of the muscle is met by a higher cellular energy status. Studies to date, however, have not investigated whether Cr loading would attenuate endurance activity-induced MHC isoform transitions from fast MHCIIb to slower MHCIIa.

The overall purpose of this thesis was to investigate the effects of chronic and acute Cr feeding combined with voluntary wheel-running and chronic low frequency stimulation (CLFS), respectively on rat skeletal muscle structure, function and metabolism. Three major studies encompass the dissertation.

The purpose of the first investigation (Chapter 2) was to assess the sensitivity and reliability of whole muscle isometric measures as indicators of whole muscle fibre-type composition. More specifically, the reliability of isometric measures of time-to-peak (TTP), half rise-time (½RT), half fall-time (½FT), twitch force (TW_f), titanic force (TET_f) and the sag ratio in several hindlimb muscles when only one limb is studied, and thereby establish the interchangeability between muscles of the right and left hindlimb. A secondary objective was to examine the sensitivity of TTP, ½RT, ½FT, TW_f, TET_f and the sag ratio, especially with regard to the ability of these measures to differentiate between two fast-twitch muscles [*i.e.*, extensor digitorum longus (EDL) and medial gastrocnemius (MG) and slow soleus (SOL)] that differ in their respective MHC-

isoform contents. Collectively the results of this study validated the use of these isometric measures to ascertain the functional impact associated with differences in patterns of MHC isoform expression and morphology across a broad physiological range.

The second investigation involved two studies (Chapters 3 and 4). The purpose of the first study was to investigate whether Cr supplementation during 13 weeks of phasic high-frequency voluntary wheel running would result in a faster MHC isoform profile in the rat mixed fast-twitch plantaris (PL), and alter its corresponding isometric contractile properties. It was anticipated that chronic Cr loading would result in qualitative and quantitative changes in patterns of MHC-isoform expression in skeletal muscles leading to altered isometric contractile properties and performance characteristics (Chapter 3). The second part of the study investigated whether chronic Cr loading combined with long-term voluntary wheel running would 1) attenuate endurance training-induced skeletal muscle fibre type transformations, 2) alter proteins involved in intracellular calcium (Ca^{2+}) regulation and 3) affect the metabolic phenotype of rat PL muscle (Chapter 4). It was hypothesized that enhancing the cellular energy status of the PL by chronic Cr feeding would allow this fast-twitch muscle to retain its fast-glycolytic phenotype after prolonged voluntary running, and also maintain high levels of parvalbumin and fast sarco/endoplasmic reticulum Ca^{2+}-ATPase isoform (SERCA1), and low levels of slow sarco/endoplasmic reticulum Ca^{2+}-ATPase isoform (SERCA2). Overall, this two-part investigation employed the highest Cr dose combined with the longest longitudinal exercise design to date reported in the literature.

The last study investigated whether acute Cr loading would attenuate CLFS-induced MHC isoform transitions, alter the corresponding isometric contractile properties, the proteins that regulate intracellular calcium and the metabolic profile of rat tibialis anterior (TA) (Chapter 5). It was hypothesized that acute Cr feeding during CLFS will maintain the fast, glycolytic MHC-based

phenotype of the fast-twitch TA muscle. And furthermore, that Cr feeding during CLFS would result in high levels of parvalbumin and fast SERCA1, and low levels of slow SERCA2 compared with a stimulated group that received no Cr.

CLFS of skeletal muscle is a good model for the study of functional relationships between induced changes in functional, metabolic and molecular characteristics (Pette and Vrbova, 1992). CLFS was chosen as an endurance-activity induced model because it is standardized and is a reproducible regimen for enhanced neuromuscular activity. CLFS activates all motor units of the stimulated muscle, even those not normally recruited in exercise. CLFS can establish a specific dose-response relationship, causes synchronous activity of all motor units and abolishes the hierarchical order of recruitment while not injuring muscle fibres (Delp and Pette, 1994 and Putman et al., 1999). CLFS imposes a higher level of activity over time than any other exercise regime and time-dependent changes in molecular, structural, and functional properties can be compared against contralateral, unstimulated muscles as an intra-animal control. CLFS is also restricted to a single muscle or small muscle groups, thus is thought to exclude interference of systemic responses (Haas 2002). The TA muscle was the primary focus of this investigation because it possesses a mixed fast-twitch MHC-based phenotype and is innervated by the common peroneal nerve which can be easily targeted by CLFS. The rat was chosen as an experimental animal because contrary to the rabbit (Maier, Gambke and Pette, 1986), the induced fast-to-slow conversion occurs without signs of fibre injury or degeneration (Putman et al., 1999). Figure 1-6 shows a schematic of the CLFS model and summarizes the CLFS-induced adaptations.

Collectively, these two distinct endurance exercise regimens (long-term voluntary wheel running and short-term CLFS) resulted in qualitatively similar fast-to-slow fibre type transitions in mixed fast-twitch rat muscle. These MHC-based phenotypic alterations agree with studies of Pette and colleagues (1992, 1996, 2001 and 2002) where it has been shown that increases in neuromuscular

activity induce the transformation of fast-twitch fibres into slower contracting and more oxidative phenotypes in rodent muscles.

Figure 1-1

The structural formula of creatine. Cr is known chemically as N-(aminoiminomethyl)-N-methyl glycine (molecular formula: $C_4H_9N_3O_2$).

Figure 1-1

CREATINE MOLECULE

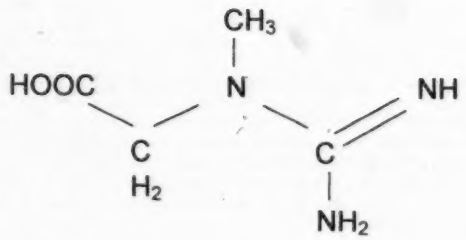


Figure 1-2

Biosynthesis of creatine.

Arginine + glycine ⇔ ornithine + guanidinoacetate (1; Rate limiting step)

Guanidinoacetate + S-adenosylmethionine ⇒ creatine +
S-adenosylhomocysteine (2)

Enzymes involved in the two-step process biosynthesis of Cr:
AGAT: L-arginine:glycine aminidotransferase
GAMT: S-adenosyl-L-methionine:N-guanidinoacetate methyltransferase

In the process, glycine is completely incorporated into the Cr backbone, whereas arginine and methionine only contribute side groups.

Figure 1-2

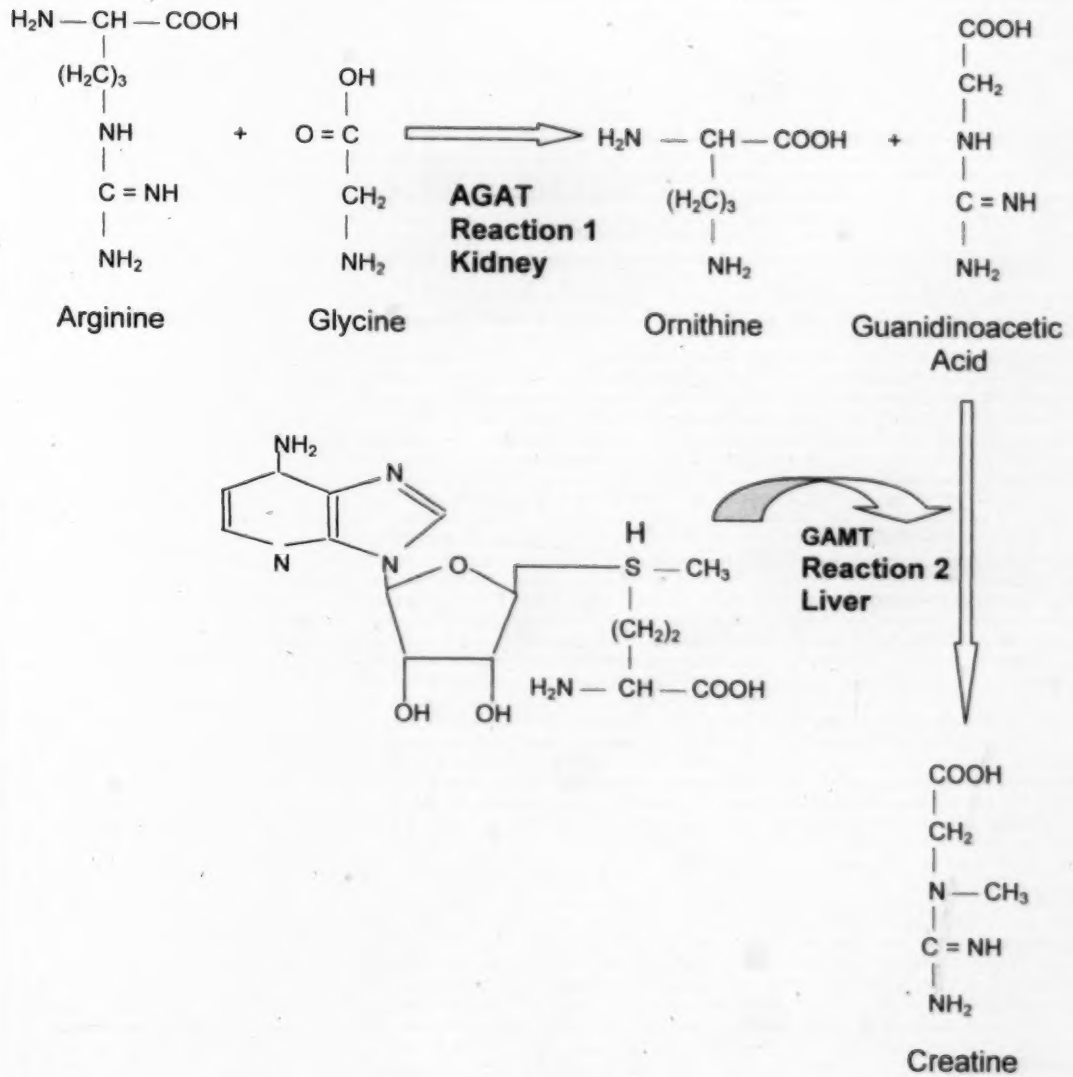
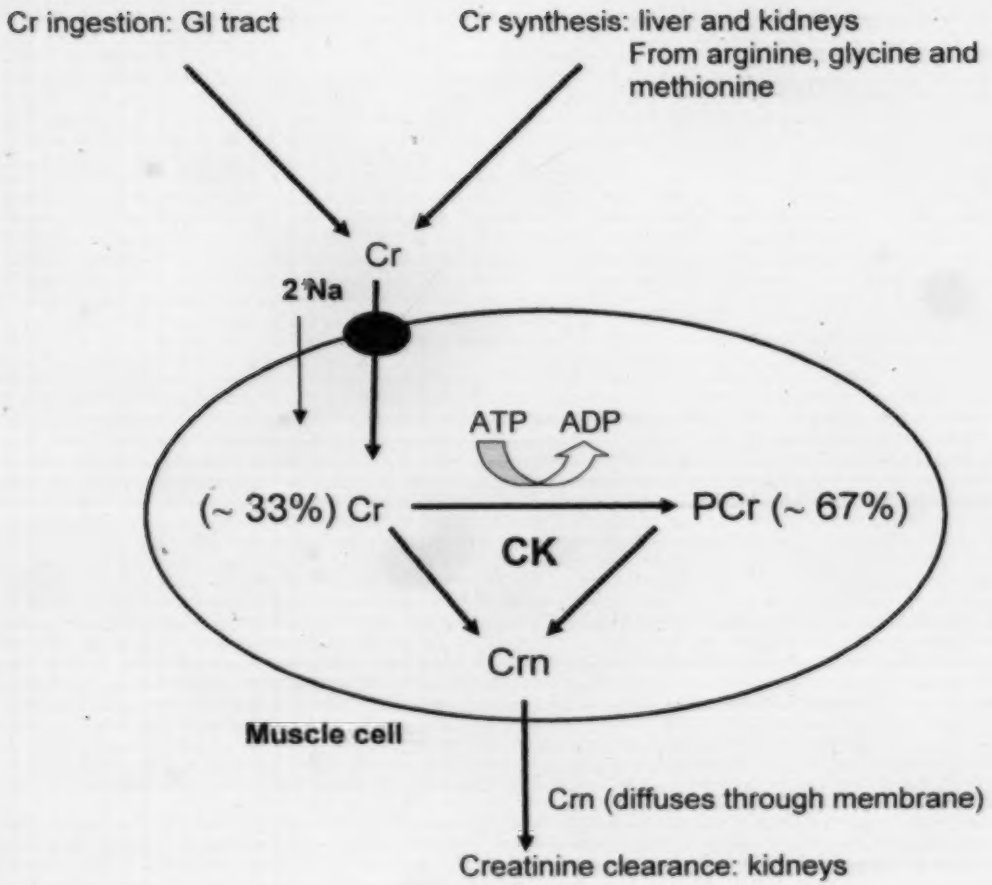

Arginine
Glycine
AGAT
Reaction 1
Kidney
Ornithine
Guanidinoacetic Acid
GAMT
Reaction 2
Liver
Creatine

Figure 1-3

Creatine transport.

Cr transport into the muscle cell is driven by the electrochemical potential gradient created by the extracellular and intracellular levels of sodium. Cr and PCr have a charge and thus become trapped within the muscle cell once transported within. Creatinine, on the other hand, has no electrical charges at physiological pH and is able to escape across the muscle cell membrane.

Figure 1-3



Cr Transporter

CK Creatine kinase

Figure 1-4

Creatine kinase reaction and creatinine formation.

Figure 1-4

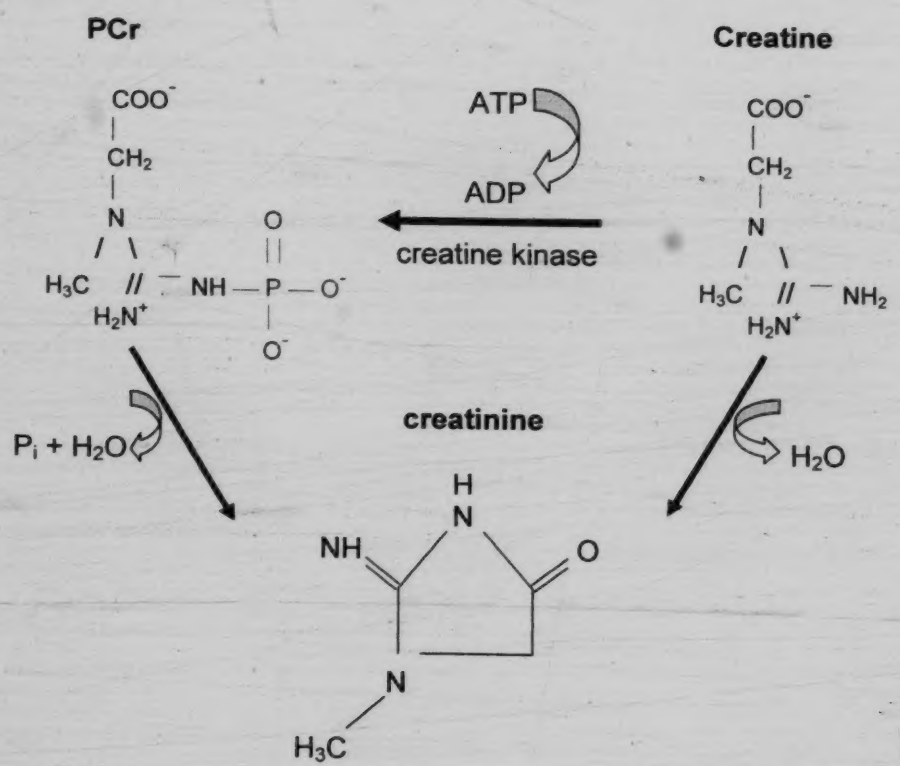
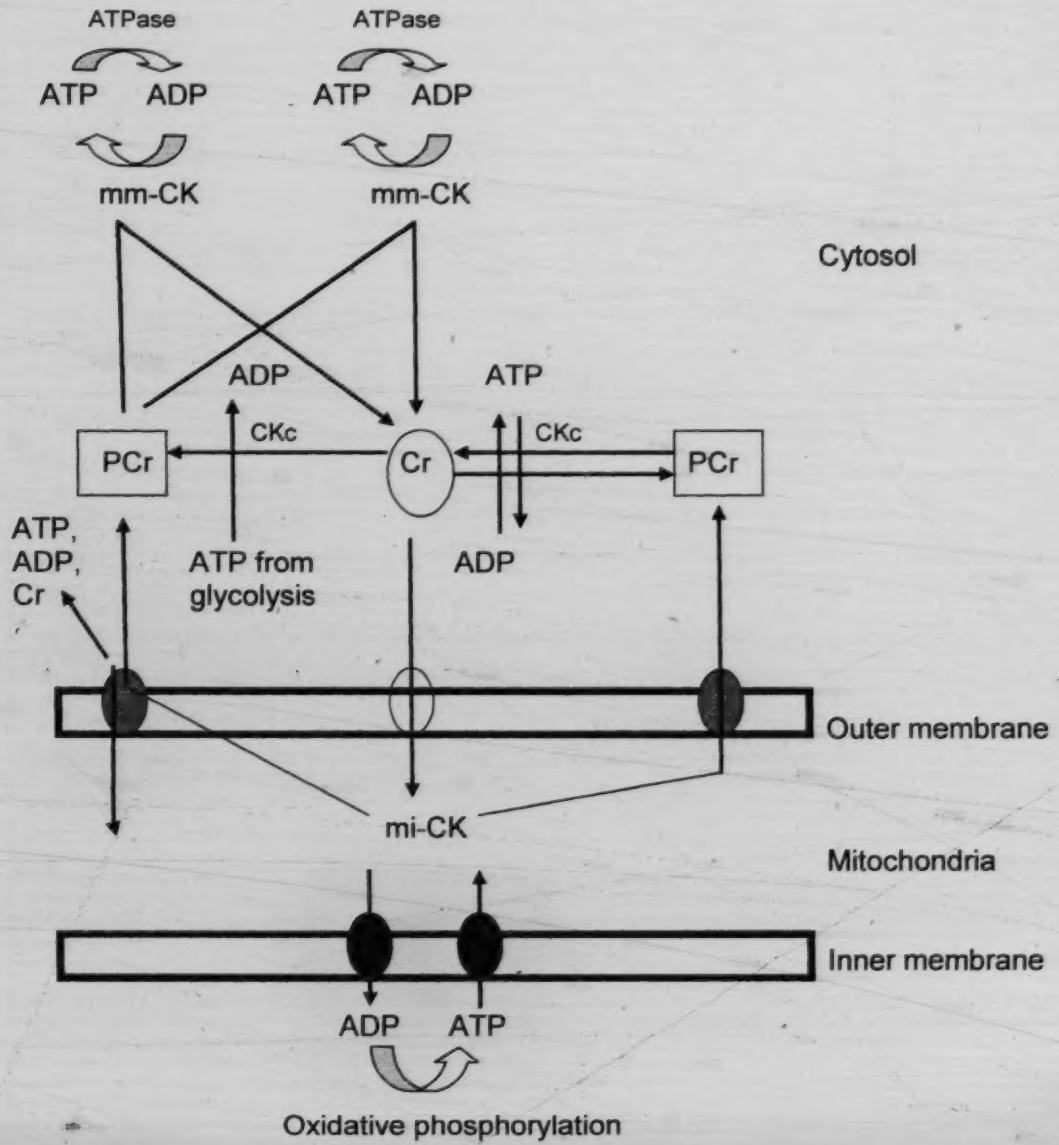

PCr
Creatine
ATP
ADP
creatine kinase
COO⁻
CH₂
N
H₃C
NH
P
O⁻
H₂N⁺
NH₂
Pi + H₂O
H₂O
creatinine
H
N
NH
O
N
H₃C

Figure 1-5

Phosphocreatine circuit shuttling system. Creatine kinase (CK) isoforms are compartmentalized: mm-CK and Ckc are the myofibril and cytosolic isoforms, while mi-CK is in the mitochondria.

Figure 1-5



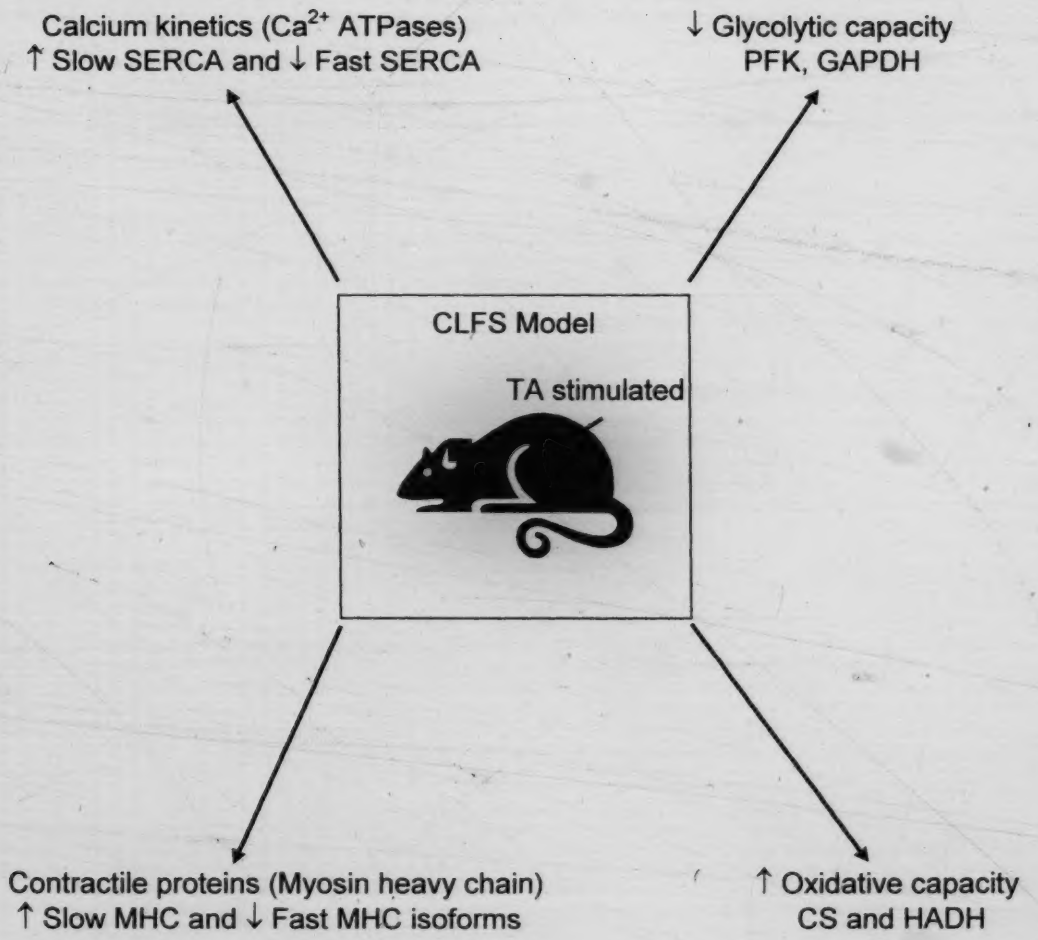
ANT: adenine nucleotide transporter

Porin channels: anion selective state and cation selective state

Figure 1-6

Schematic of the chronic low frequency stimulation model. In the rat, a portable external stimulator can be fixed to the back of the animal and the electrodes are passed subcutaneously and sutured to the sides of the peroneal nerve in the hindlimbs: this nerve innervates the tibialis anterior (TA) and the extensor digitorum longus (EDL) muscles. In the contralateral limb of the animal is the unstimulated muscle, which acts as the internal control. The duration of the stimulation bouts per day and period of stimulation in days are variable. CLFS produces a wide range of cellular adaptations including modifications of the contractile proteins (myosin heavy chain proteins), changes in the SR Ca^{2+} handling kinetics and changes in the enzyme activities involved in oxidative metabolic pathways in the mitochondrion and glycolytic capacity.

Figure 1-6


Calcium kinetics (Ca^{2+} ATPases)
↑ Slow SERCA and ↓ Fast SERCA
↓ Glycolytic capacity
PFK, GAPDH
CLFS Model
TA stimulated
Contractile proteins (Myosin heavy chain)
↑ Slow MHC and ↓ Fast MHC isoforms
↑ Oxidative capacity
CS and HADH

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[1]CHAPTER 2: Reliability and sensitivity of isolated isometric function measures in rat muscles composed of different fibre types

2.1 INTRODUCTION

Skeletal muscle is a heterogeneous, post-mitotic tissue that displays a considerable capacity to alter its structural phenotype and corresponding functional properties. Alterations in muscle contractile activity, loading conditions, thyroid status and exposure to pharmacological agents have, for example, been shown to induce changes in the expression patterns of MHC isoforms, in proteins regulating excitation-contraction (E-C) coupling and in the metabolic profile (Pette and Staron 1997; Pette and Staron 2000; Caiozzo et al. 1996; Irintchev et al. 2002; Larsson et al. 1994). The pattern of MHC-isoform expression correlates very highly with functional parameters that reflect contraction speed (Talmadge et al. 2002; Galler et al. 1996; Bottinelli et al. 1991 and 1994a; Galler et al. 1994). Owing to its close coordinate expression with proteins regulating E-C coupling (Hämäläinen and Pette 1997) and metabolism (Dunn and Michel 1997), the MHC profile also has considerable value as a predictor of relaxation rate, generation of force and fatigability. However, the sensitivity and reliability of whole muscle isometric measures as indicators of whole muscle fibre-type composition remain to be investigated.

Isometric measures used to assess whole muscle contraction speed commonly include the time to peak tension development (TTP) and the half rise-time (½RT) (Stein et al. 1982; Gordon and Stein 1985), and are primarily determined by the pattern of MHC isoforms expressed. The rate of relaxation, on the other hand, is typically measured by the half fall-time (½FT). In fast twitch fibres, this property is partly dependent on the expression of the fast Ca^{2+}-ATPase (SERCA1) isoform and parvalbumin (Schwaller et al. 1999; Hämäläinen

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[1] A version of this chapter has been published. Gallo M, Gordon T, Tyreman N, Shu Y and Putman CT. *Experimental Physiology*. 89(5): 583-592, 2004.

and Pette 1997). By comparison, in slow fibres, the ½FT is related to the expression of the slow SERCA2a isoform (Hämäläinen and Pette 1997). Twitch (TW_f) and tetanic (TET_f) force production are frequently used to assess whole muscle strength; these measures reflect aspects of the muscle phenotype that include fibre type composition (i.e. myosin ATPase activity), architecture, and the number of contractile units in parallel and series (Rafuse et al. 1997). At the motor unit level, the "sag" and force generated during subtetanic stimulation varies systematically with these other isometric measures (Gordon et al. 1997; Rafuse et al. 1997; Tötösy de Zepetnek et al. 1992). Thus it follows that, at the whole muscle level, the sag ratio should reflect the average motor unit properties, and may be useful to investigate functional differences associated with fatigue between muscles composed of different fibre types.

The primary purpose of this study was to assess the reliability of isometric measures of TTP, ½RT, ½FT, TW_f, TET_f and the sag ratio in several hindlimb muscles when only one limb is studied and thereby establish the interchangeability between muscles of the right and left hindlimb. A secondary objective was to examine the sensitivity of TTP, ½RT, ½FT, TW_f, TET_f and the sag ratio, especially with regard to the ability of these measures to differentiate between two fast-twitch muscles (i.e., EDL and MG) that differ in their respective MHC-isoform contents. The range of our analysis was expanded to also include the slow SOL muscle. The results of this investigation show that small variations in the MHC-isoform contents of fast-twitch rodent muscles are reflected in whole muscle TTP, ½RT and ½FT. Measures of TW_f, TET_f and sag also varied systematically with MHC isoform contents but displayed considerable dependence on other morphological properties. Overall the right and left legs were interchangeable, and displayed a large range of reliability when only one hindlimb was studied.

2.2 MATERIALS AND METHODS

2.2.1 *Animals and surgery*

Twelve adult male Sprague-Dawley rats (595 ± 33 g body weight) were obtained from the local animal facility at the University of Alberta and used in the present study. All experiments were completed in accordance with the guidelines of the Canadian Council for Animal Care and received ethical approval from the University of Alberta. All animals were maintained under controlled environmental conditions (22°C and 12-h alternating light and dark cycles), and received standard chow and 5% (w/v) dextrose solution ad libitum.

Surgery was performed under general anaesthesia (45 mg/kg sodium pentobarbital IP, MTC Pharmaceutical, Cambridge, Ontario) according to Tam et al. (2001). Briefly, an indwelling catheter (PE 50) was placed in the external jugular and the trachea was cannulated for mechanical ventilation if necessary. Additional Somnotol (diluted 1:5) was administered intravenously in a saline solution containing 5% (w/v) glucose, as required. Incisions were made along the dorsum of the right and left hindlimbs, and the EDL, SOL and MG muscles were exposed, and the TA was denervated. A silastic nerve cuff embedded with 2 multistranded stainless steel wires was placed around the sciatic nerve for electrical stimulation. The distal tendons of each muscle were subsequently isolated and individually secured with 2.0 silk (metric 3) to a Kulite strain gauge (model KH-102). Extreme care was taken to ensure that the arterial supply and venous drainage remained intact during the isolation of each muscle. Skin incisions were then sutured before beginning functional measurements. Animals were placed in a prone position and secured with clamps at the knees and ankle joints. Core body temperature was maintained at 39^{0}C with a heating pad, and continuously monitored with a rectal probe. Animals were euthanized and muscles were collected, snap-frozen in liquid nitrogen (-196 °C) and stored at

-80 °C until analysed. The mean muscle weights of the SOL, EDL and MG were 0.235 ± 0.017 g, 0.248 ± 0.022 g and 1.405 ± 0.113 g, respectively.

2.2.2 *Functional measurements*

Isometric muscle function measures were completed according to Tam et al. (2001). Before each series of recordings, the optimal resting length (L_o) required to generate maximum force was determined for each muscle. Maximum twitch (TW_f: mN) and tetanic (TET_f: mN) forces were sequentially recorded in the right and then left EDL, SOL and MG. TW_f was determined as the average of peak forces generated by five individual twitches elicited at 1 Hz. Time-to- peak tension (TTP: ms) was determined as the time elapsed from time 0 to peak force production. The half-rise time (½RT: ms) was calculated as the time from the onset of contraction to 50% of the maximum force produced. The half-fall time (½FT: ms) was recorded as the time required for the twitch peak force to decay by 50%. The presence of whole muscle "sag" was calculated as the ratio of final-to-initial force generated from an unfused tetanic contraction using a stimulus interval of 1.25×TTP for 800 msec. Traditionally the sag ratio has been a used to classify single motor units as "slow" if the ratio is ≥1.0 or "fast" if the ratio is <1.0 (Gordon et al. 1997; Rafuse et al. 1997). In the present study we investigated the degree to which the sag test may also reflect changes in the average motor unit composition at the whole muscle level. TET_f was determined as the force recorded in response to 21 stimulus pulses (100 Hz) given at an interstimulus interval of one-third of the muscle contraction time (Gordon and Stein 1995; Tötösy de Zepetnek et al. 1992). In addition, the TW_f and TET_f measures were normalized by dividing the individual twitch and tetanic forces by the averaged wet muscle weights. Lastly, the twitch-to-tetanus (TW_f : TET_f) ratio was calculated by dividing the twitch force by the tetanic force. All measurements were amplified, viewed on an oscilloscope, averaged using a PDP11-lab computer and stored on disks.

2.2.3 *Electrophoretic analyses myosin heavy chain.*

MHC isoforms were analyzed according to the methods described by Hämäläinen and Pette (1996). Muscles were homogenized on ice in a buffer containing 100 mM $Na_4P_2O_7$ (pH 8.5), 5 mM EGTA, 5 mM $MgCl_2$, 0.3 M KCl, 10 mM DTT and 5 mg/ml of a protease inhibitor cocktail. Samples were subsequently stirred for 30min on ice, centrifuged at 12,000 × *g* for 5min at 4°C, diluted 1:1 with glycerol, and stored at -20 °C until analyzed. Prior to gel loading, muscle extracts were diluted to 0.2 μg / μl in Laemmli-lysis buffer and boiled for 7min. Five μl from the resulting MHC extracts were electrophoresed in duplicate for 24 or 48 hrs (275 V and 12 °C) on 7% (w/v) PAA gels containing glycerol under denaturing conditions. Gels were immediately fixed, and MHC isoforms were detected by silver staining (Oakley et al. 1980) and evaluated by densitometry. The gel running time was varied to maximize MHC-isoform separation for the various muscles.

2.2.4 *Statistical Analyses*

Data are presented as mean ± SEM. Absolute reliability (R_{Ab}) was defined as the consistency or repeatability of a measure, expressed as a ratio of the variance of the "true score" to the variance of the measured score, and was calculated as follows:

$$R_{Ab} = \sigma_R^2 / [\sigma_R^2 + (\sigma_L^2 / n_L) + (\sigma_{SD,e}^2 / n_D)]$$

σ^2_R is the estimate of the rat variance component, σ_L^2 represents the estimate of the leg variance component, n_L (number of legs) = 1, n_D (number of days) = 1, and $\sigma_{SD,e}^2$ is the estimate of the residual variance component. σ^2_R, σ_L^2 and $\sigma_{SD,e}^2$ were calculated by One-way Analysis of Variance (ANOVA). Differences between group means of the isometric functional measures and R_{Ab} were assessed using an independent sample Student's t-test. Right and left legs were

also compared by individual correlation analyses (r) where the SOL, MG and EDL muscles were averaged, each with a sample size of 10 (total n=30). Differences between MHC isoform composition group means were assessed using a One-Way ANOVA. Isometric measures were evaluated against each of the various MHC isoforms by multiple regression analysis according to Talmadge et al. (2002); correlation coefficients derived from this analysis (R) are reported (n=36). Differences were considered significant at $P < 0.05$.

2.3 RESULTS

2.3.1 *Isometric functional measures: force and speed parameters*

The slow SOL displayed the longest TTP at 55.0 ± 3.1 ms, followed by the EDL at 39.5 ± 0.8 ($P < 0.00003$), and the faster contracting MG at 25.1 ± 0.4 ($P < 0.01$). The MG was considerably faster ($P < 0.01$) than EDL (Figure 2-1A). The SOL also displayed the longest ½RT at 32.1 ± 2.04 ms ($P < 0.01$) (Figure 2-1A). Additionally, the ½RT of the EDL (19.2 ± 0.4) was 24% slower than the MG (14.6 ± 0.3, $P < 0.01$). The ½FT of the SOL was greater than 2-fold longer (57.3 ± 3.6 ms, $P < 0.01$) compared to all other muscles; the ½FT of the remaining muscles followed the order EDL (23.5 ± 1.5) > MG (12.3 ± 0.5) (Figure 2-1, $P < 0.01$).

The SOL generated less than half of the TW_f (693 ± 57.3 mN, $P < 0.01$) observed in the EDL (1531 ± 98) and MG (1600 ± 99) (Figure 2-2A). Similarly TET_f was also lowest in the SOL (2920 ± 152 mN) compared to all other muscles ($P < 0.007$). The EDL (3603 ± 179) generated ~30% less TET_f than MG (5403 ± 191) (Figure 2-2B, $P < 0.01$). However, when the TW_f was standardized for muscle weight the EDL produced the most force (6.17 ± 0.45 mN/mg, $P < 0.03$) followed by the SOL (2.84 ± 0.28, $P > 0.75$), and lastly the MG (1.14 ± 0.08) (Figure 2-2D, $P < 0.01$). The EDL generated the greatest normalized TET_f (14.5 ± 0.80 mN/mg, $P = 0.07$) whilst values for the SOL and MG were considerably

less (12.4 ± 0.81 and 3.8 ± 0.14, respectively) (Figure 2-2E). The TW_f:TET_f ratio of the selected muscles was as follows: EDL (0.44 ± 0.14) > MG (0.26 ± 0.018) > SOL (0.25 ± 0.006) (Figure 2-2C). The whole muscle SOL sag ratio was 2.6-5.4 fold greater than observed for the mixed fast-twitch EDL and PL muscles ($P < 0.01$). The sag ratio for the EDL (0.83 ± 0.02) differed from the MG by 53% (Figure 2-2F, $P < 0.01$).

2.3.2 Myosin Heavy Chain content

The various MHC isoform contents within the different muscles are qualitative represented in the gels of Figure 2-3, and quantitatively illustrated in Figure 2-4. MHCI isofrom content (Figures 2-4A) was most abundant in the SOL where it comprised 89.5 ± 2.0% of the total MHC content and was 13-30 fold greater than in the other muscles studied ($P < 0.01$). Within the mixed fast twitch EDL, the MHCI content was 3.1 ± 0.4%. By comparison the MHCI content was approximately 2-fold greater ($P < 0.002$) in the MG reaching 5.9 ± 0.4%.

The muscles studied displayed a narrow range of MHCIIa isoform content (Figure 2-4B). The SOL (10.1 ± 1.5%), EDL (9.9 ± 0.7%) and MG (7.3 ± 1.2%) expressed similar proportions of MHCIIa. The MHCIId(x) isoform content of the EDL (33.3 ± 1.6%) was greater than observed in the MG (25.9 ± 2.5%) (Figure 2-4C, $P < 0.05$). By comparison the MHCIIb isoform content (Figure 2-4D) in the EDL was 53.7 ± 1.7% of total MHC, and was lower than that of the MG (60.9 ± 2.1%). Although they differed by only ~7%, the EDL and MG were significantly different ($P < 0.02$). The MHCIId(x) or MHCIIb isoforms were not detected in SOL.

2.3.3 *Correlation Analyses*

Table 2-1 summarizes the correlation coefficients derived from multiple regression analysis (R) of MHC isoforms and isometric functional measures. High negative correlations were observed in the TTP, ½ RT, ½ FT and sag ratio for MHCIId/x, MHCIIb ($P < 0.01$), but not for the MHCIIa isoform. Conversely, the TTP, ½ RT, ½ FT and the sag ratio displayed high positive correlations with MHCI. The TW_f and TET_f measures displayed a wide range of correlation coefficients (R), most of which were statistically significant. Normalized TW_f was not highly correlated to any of the MHC isoforms. R-values for normalized TET_f were also low, with the exception of MHCIId/x ($R = -0.38$, $P < 0.05$). The TW_f:TET_f ratio showed low correlations, except in the case of MHCI and MHCIId/x were the R-values were -0.34 and 0.49 ($P < 0.05$).

2.3.4 *Intra-animal variation*

Differences between the right and left legs were evaluated by performing a paired dependent sample Student's t-test. Isometric functional measures were not different between the right and left legs (Figure 2-5A-F). The lone exception was TET_f, where the left SOL was 17% lower than the right ($P < 0.003$) (Figure 2-5E). In addition, individual correlation coefficient analyses (r) were completed to further compare the left and right legs (Figure 2-5). All measures showed high correlations between the left and right legs.

To further examine the variance components within animals, the absolute reliability (R_{Ab}) was calculated for each isometric functional measure (Figure 2-6). R_{Ab} is the ratio of animal variance-to-total variance; the latter incorporates animal variance, single leg variance and residual variance. Thus the resultant R_{Ab} is inversely proportional to the magnitude of the leg variation and the variance associated with measurement. The reliability of a measure was considered high if $R_{Ab} > 0.60$ and moderate if $0.2 \leq R_{Ab} \leq 0.60$. R_{Ab} values less than 0.2 were

considered low by comparison. In the present study, the TTP, ½ RT, ½ FT, TW_f, TET_f and sag ratio displayed a range of R_{Ab} for the individual muscles studied (Figure 2-6 A and 2-6B). However, when averaged across all of the muscles, the R_{Ab} was high for TTP, ½ RT, ½ FT and TW_f, and within the moderate range for TET_f and sag ratio (Figure 2-6 C and 2-6D).

Figure 2-1

Isolated isometric speed parameters for selected hindlimb muscles. "a" different from all other muscles. Differences between group means were assessed using an independent sample Student's t-test (both left and right legs were included, n=20 for each muscle).

Figure 2-1

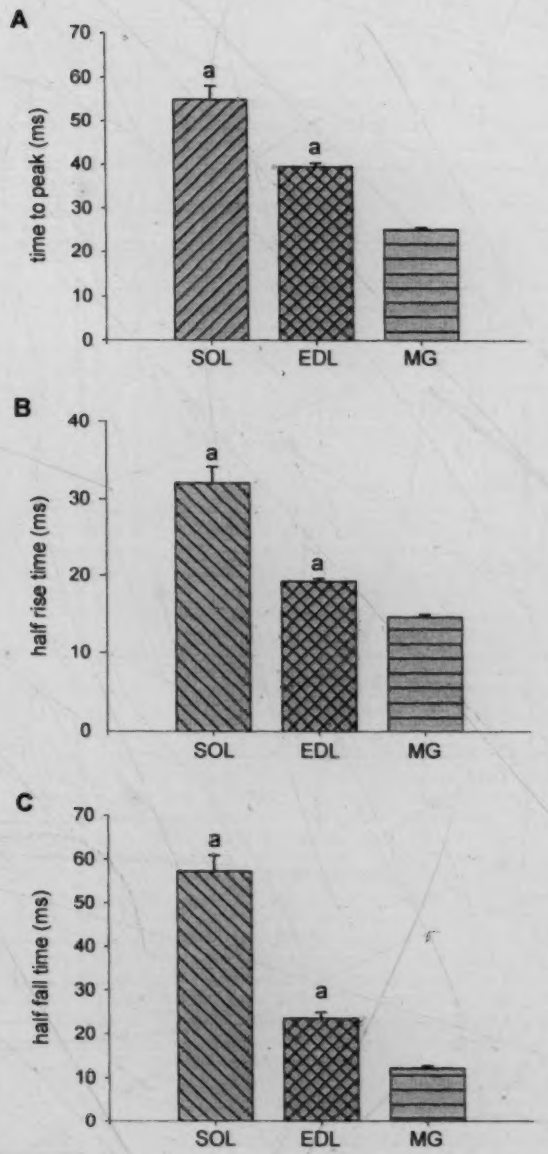

A
time to peak (ms)
70
60
50
40
30
20
10
0
a
a
SOL
EDL
MG
B
half rise time (ms)
40
30
20
10
0
a
a
SOL
EDL
MG
C
half fall time (ms)
70
60
50
40
30
20
10
0
a
a
SOL
EDL
MG

Figure 2-2

Select isolated isometric functional measures for hindlimb muscles. "a" different from all other muscles. Differences between group means were assessed using an independent sample Student's t-test (both left and right legs were included, n=20 for each muscle).

Figure 2-2

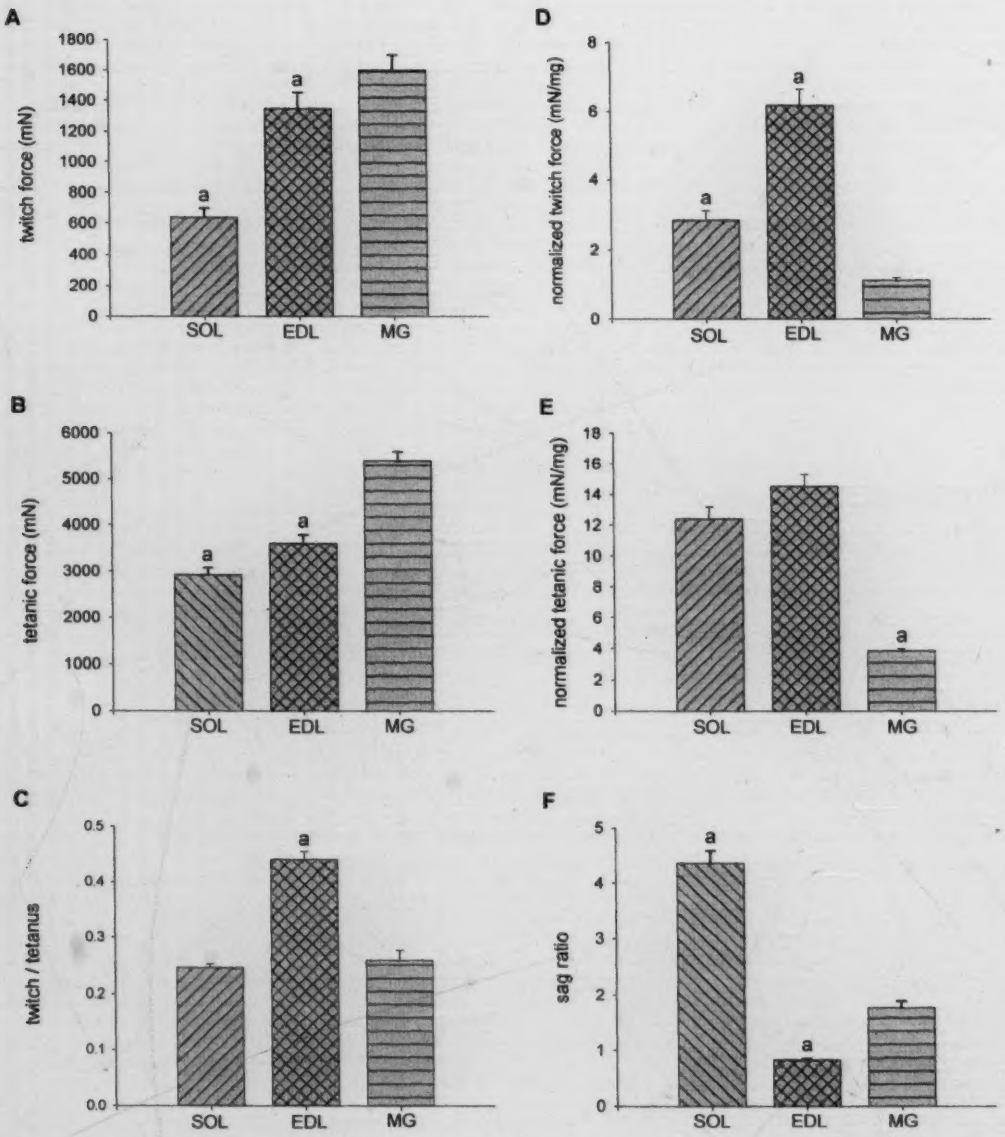

A
twitch force (mN)
1800
1600
1400
1200
1000
800
600
400
200
0
a
a
a
SOL
EDL
MG
D
normalized twitch force (mN/mg)
8
6
4
2
0
a
a
SOL
EDL
MG
B
tetanic force (mN)
6000
5000
4000
3000
2000
1000
0
a
a
SOL
EDL
MG
E
normalized tetanic force (mN/mg)
18
16
14
12
10
8
6
4
2
0
a
SOL
EDL
MG
C
twitch / tetanus
0.5
0.4
0.3
0.2
0.1
0.0
a
SOL
EDL
MG
F
sag ratio
5
4
3
2
1
0
a
a
SOL
EDL
MG

Figure 2-3

Electrophoretic method used to quantify myosin heavy chain isoform composition in select rat hindlimb muscles. The SOL and MG were electrophoresed for 24hrs, while optimal separation for the EDL was achieved after 48 hrs of electrophoresis.

Figure 2-3

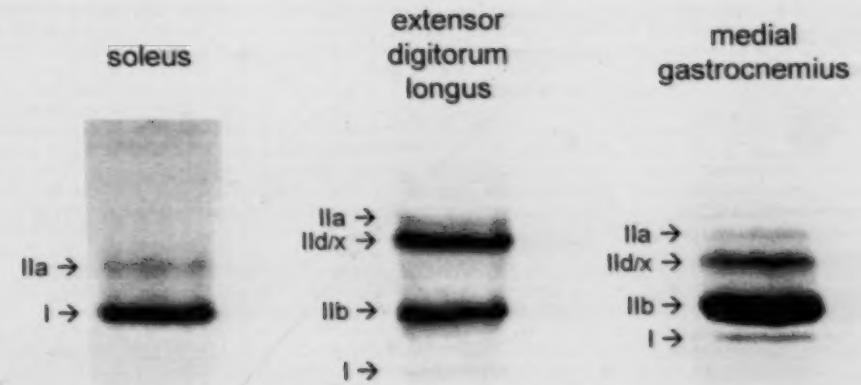

soleus
extensor digitorum longus
medial gastrocnemius
IIa →
I →
IIa →
IId/x →
IIb →
I →
IIa →
IId/x →
IIb →
I →

Figure 2-4

Myosin heavy chain isoform composition and phenotypic factor of select rat hindlimb muscles. "a" , different from all other muscles. Differences between group means were assessed using a One-Way ANOVA (both left and right legs were included, n=20 for each muscle).

Figure 2-4

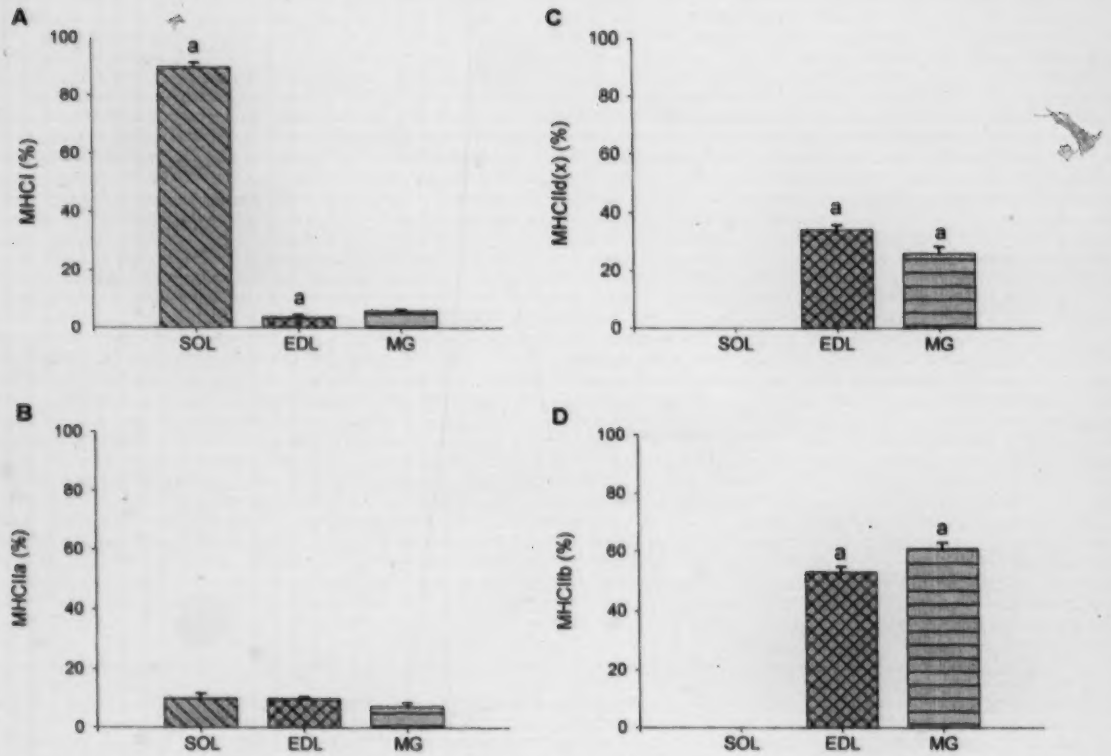

A
MHCI (%)
100
80
60
40
20
0
a
a
SOL
EDL
MG
C
MHCIId(x) (%)
100
80
60
40
20
0
a
a
SOL
EDL
MG
B
MHCIIa (%)
100
80
60
40
20
0
SOL
EDL
MG
D
MHCIIb (%)
100
80
60
40
20
0
a
a
SOL
EDL
MG

Table 2-1

Correlation coefficients derived from multiple regression analyses of MHC isoforms and isometric functional measurements (n=36)

Isometric Measure	MHC Isoforms			
	MHCI	MHCIIa	MHCIId/x	MHCIIb
TW_f	-0.61 ‡	-0.03	0.65 ‡	0.54 *
TTP	0.72 ‡	0.03	-0.52 *	-0.74 ‡
½ RT	0.79 ‡	0.15	-0.63 ‡	-0.82 ‡
½ FT	0.89 ‡	0.06	-0.78 ‡	-0.87 ‡
TET_f	-0.53 ‡	-0.29	0.43 +	0.58 *
sag ratio	0.86 ‡	0.12	-0.85 ‡	-0.80 ‡
Normalized TW_f	-0.09	0.20	0.31	-0.08
Normalized TET_f	0.25	0.19	-0.005	-0.38 †
$TW_f : TET_f$	-0.34 †	0.20	0.49 †	0.20

Data are correlation coefficients derived from multiple regression analyses (R). MHC isoforms are the dependent variables while the isometric variables are the independent variables examined. ‡, indicates $P < 0.0001$; *, indicates $P < 0.001$; +, indicates $P < 0.01$; †, $P < 0.05$.

Figure 2-5

Comparison of isolated isometric functional measures between muscles of the right and left hindlimbs. " * ", left leg is different from right leg. Differences between group means were assessed using a dependent sample Student's t-test. All correlations coefficients were highly significant, $P < 0.01$.

Figure 2-5

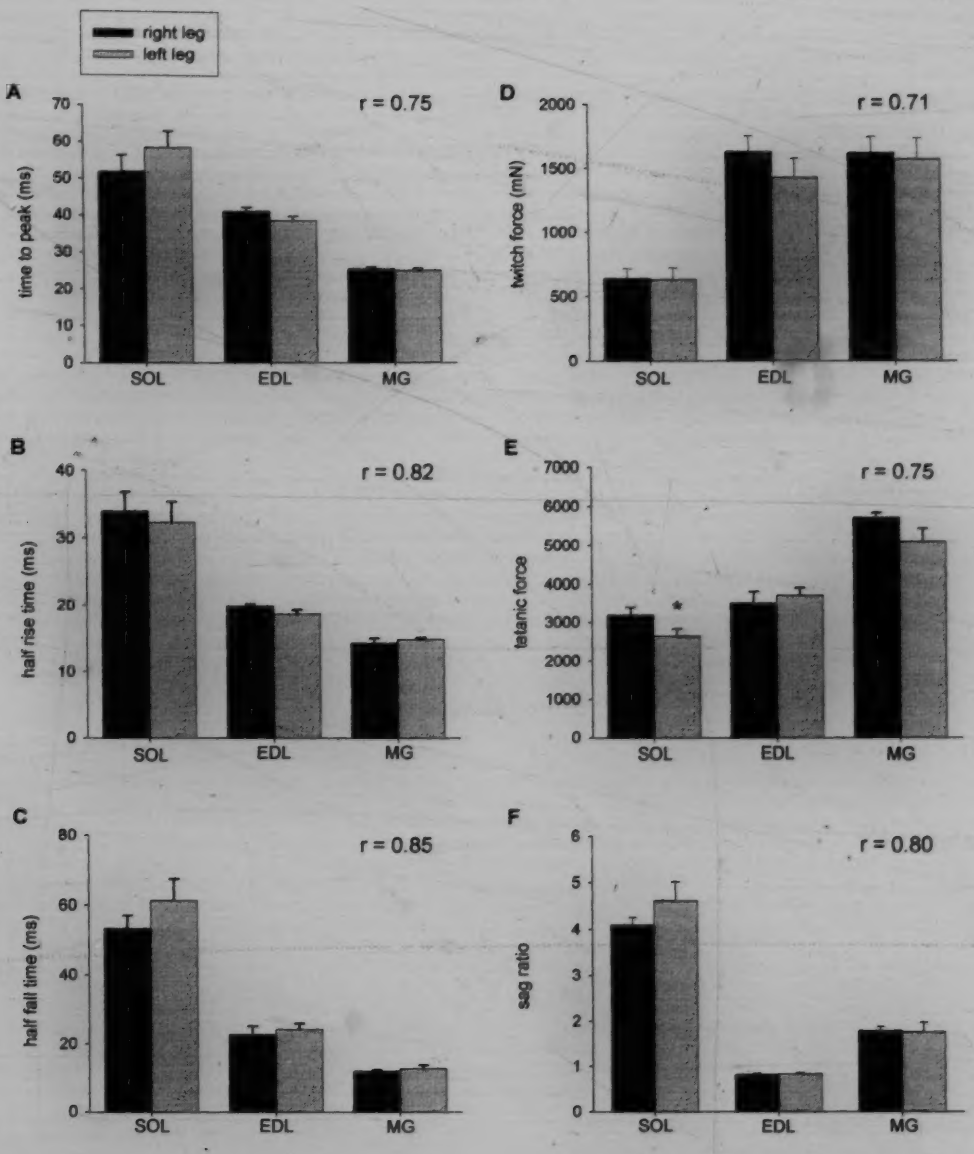
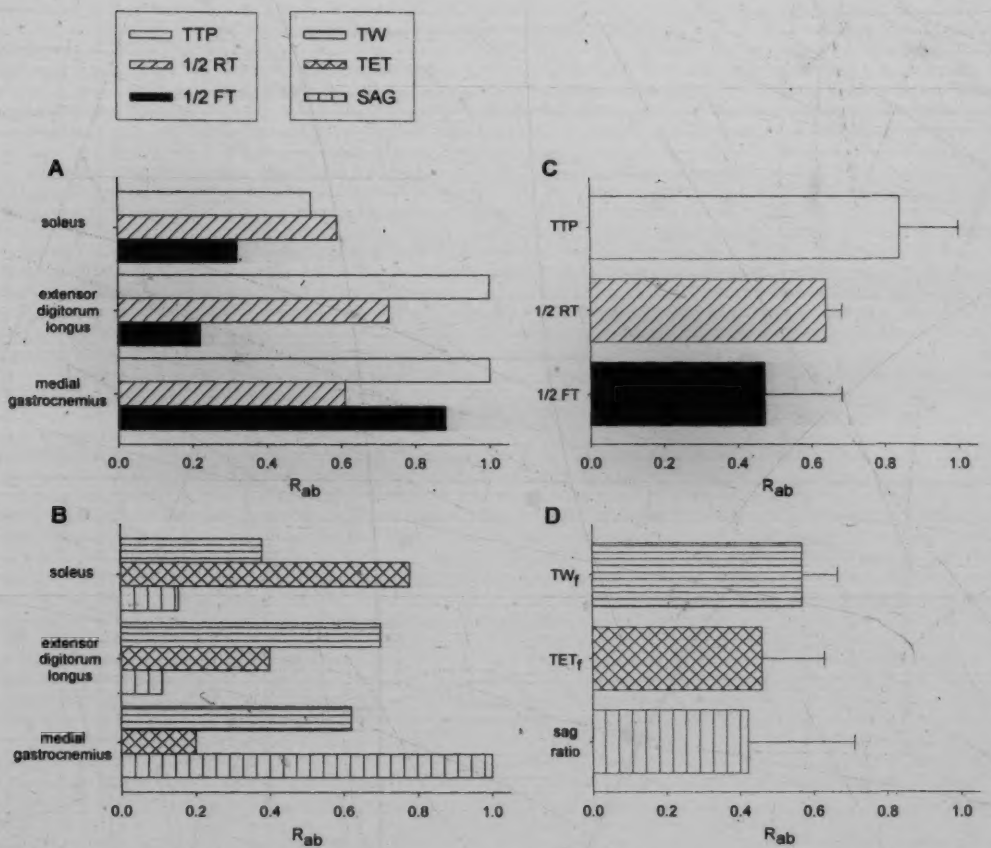

right leg
left leg
A
time to peak (ms)
r = 0.75
SOL
EDL
MG
D
twitch force (mN)
r = 0.71
B
half rise time (ms)
r = 0.82
E
tatanic force
r = 0.75
*
C
half fall time (ms)
r = 0.85
F
sag ratio
r = 0.80

Figure 2-6

Absolute reliability scores of isometric functional measures for selected hindlimb muscles. Differences between group means were assessed using an independent sample Student's t-test.

Figure 2-6


TTP
1/2 RT
1/2 FT
TW
TET
SAG
A
soleus
extensor digitorum longus
medial gastrocnemius
0.0
0.2
0.4
0.6
0.8
1.0
R_{ab}
C
TTP
1/2 RT
1/2 FT
R_{ab}
B
soleus
extensor digitorum longus
medial gastrocnemius
R_{ab}
D
TW_f
TET_f
sag ratio
R_{ab}

2.4 DISCUSSION

The TTP, ½RT and ½FT of isometric twitch contractions describe the speed characteristics of whole muscle, while the TW_f and TET_f characterize the force parameters; for each of these measures our data were similar to previous reports for rat (Close 1969; Petrofsky and Fitch 1980) and other mammalian muscles (Gordon and Stein 1985; Gillespie et al. 1986; Gordon et al. 1997). Because the "sag test" has been used to reliably identify individual motor unit types (Gordon et al., 1997), we also sought to determine if this test could predict the average motor unit composition of whole muscle, as reflected by the MHC-isoform profile. These isometric measures were highly correlated with the prevailing pattern of MHC isoform expression. Although only the speed parameters are known to possess a causal relationship with MHC, the remaining functional properties varied systematically with the MHC isoform profile, and thus displayed significant predictive value.

2.4.1 *Isometric functional measures in relation to myosin heavy chain isoform content*

The relationship between the pattern of MHC isoform expression and functional properties of single fibres has been well established in vitro (Metzger et al., 1985; Bottinelli et al. 1991; Bottinelli et al. 1994b), where measures that reflect fibre contraction time have been shown to be directly proportional to myosin-ATPase activity (Bottinelli et al. 1994b). On the motor unit level, the sag ratio is inversely proportional to myosin-ATPase activity owing to its close coordinate relationship with other aspects of fibre phenotype, such as the capacity to generate energy via oxidative pathways (Larsson et al. 1991; Gordon 1995; Rafuse et al. 1997). Measures of TW_f and TET_f are also proportional to myosin-ATPase activity; TTP, ½RT, TW_f and TET_f are further dependent on aspects of muscle architecture that include cross-sectional area (Segal et al., 1986), muscle and fibre lengths (Huijing et al., 1989; Huijing et al., 1994) and

fibre pennation angle (Woittiez et al. 1984; Huijing et al. 1989; Huijing et al. 1994).

The design of our study allowed us to examine isometric contractile force, speed and fatigability in slow- and fast-twitch skeletal muscles within the same rat. With regard to the fast-twitch muscles, a modest difference in MHC content, reflecting different fiber type proportions, was detectable by measures of contractile speed. The MG was considerably faster than the EDL (Figure 2-1), which was primarily related to the 7% greater MHCIIb isoform content and 7% lower MHCIId(x) isoform content (Figure 2- 4). Although the MG is known to posses a greater fibre pennation angle compared to the EDL (i.e., mean ± SD: $10.0^{o} \pm 1.41$ vs. $20.4^{o} \pm 5.53$), the mean fibre length of these two muscles are similar (Huijing et al., 1994); since TTP and ½RT vary inversely with the angle of pennation, the faster contraction time of the MG must have resulted from different patterns of MHC-isoform expression between these fast-twitch muscles. This finding indicates that TTP and ½RT are sensitive enough to detect comparatively modest differences in the patterns of MHC-isoform expression. It is also clear from the present study that the ½FT varied systematically with these speed parameters and was similarly sensitive. Additionally, a comparison of these two fast-twitch muscles with the slow SOL muscle further demonstrates considerable utility across a broad physiological range.

Similarly the force characteristics are, to varying degrees, dependent on muscle architectural structure and MHC-isoform content. The graded phenomena observed for the TW_f and TET_f measures (Figure 2-2A and B) can be attributed to differences in muscle mass (i.e., sarcomeres in parallel), fibre length (i.e., sarcomeres in series), fibre pennation angle as well as the pattern of MHC-isoform expression. However, when these measures were normalized to muscle mass, the MG obviously produced less twitch and tetanic force (Figure 2-2D and E). The greater normalized TW_f of the EDL compared to SOL undoubtedly resulted from the greater proportion of fast MHC-isoforms present in

the former, as the architectural differences between these two muscles are minimal (Woittiez et al. 1984). In contrast, normalized TW_f of the MG was approximately 6-fold lower than the EDL, which appears to be primarily due to its much greater pennation angle (Woittiez et al. 1984). The inherent architectural differences between the MG and EDL muscles probably also form the basis for a similar pattern of low efficiency with regard to normalized TET_f displayed by the former.

While the utility of the sag ratio is well established at the motor unit level (Gordon et al. 1997; Rafuse et al. 1997), the utility of this measure at the level of whole muscle is limited. In the present study the sag ratio of the MG was not strictly correlated with its known motor unit profile, based on the patterns of MHC-isoform expression. When compared with the fast EDL (i.e., $\leq$ 1.0) and slow SOL (>> 1.0), which displayed values within their expected ranges, the larger than expected values observed for the faster MG probably reflects the impact of structural or metabolic differences compared with the EDL. Thus when applied to whole muscle, measures of sag appear to be suitable only to compare muscles of the same type.

2.4.2 *Reliability of isometric measures*

The R_{Ab} is the ratio of the animal variance to the total variance. In the present study, we minimized animal variance by maximizing the homogeneity of our sample population, which allowed us to expose the comparatively greater variance associated with select isometric functional measurements. Standard tests of statistical significance indicated that these measures were reliable. The Student's *t*-test for dependent means, for instance, showed that the left and right legs were not different for any of the fast-twitch muscles studied, and most measures of the slow SOL (Figure 2-5). Moreover, correlation analyses comparing mean values for the right and left legs revealed these measures were highly correlated for all muscles (Figure 2-5, $P < 0.01$). Estimates of the

individual R_{Ab}, however, revealed that these isometric measures were more variable than previously assumed. The MG displayed the highest overall reliability for all measures (i.e., 0.62 - 1.00) except TET_f, which was low (i.e., 0.2). The EDL also displayed high reliability for TTP, TW_f and ½ RT (0.70 – 1.00), but was not reliable for sag ratio, ½ FT or TET_f (0.1-0.4). In contrast the TTP, ½ RT and TET_f (0.52-0.80) proved reliable for the SOL; however, the sag ratio, ½ FT and TW_f displayed low R_{Ab} (0.1-0.38).

When, however, the individual functional tests were examined across all muscles studied, the mean reliability was moderate to high for all measures. Thus the discrepancies displayed between standard tests of statistical significance and calculated R_{Ab} suggests that caution must be exercised when making inferences about data obtained from isometric measures of TTP, ½RT, ½FT, TW_f, TET_f and the sag ratio. In this regard, measures of the same muscle on both hindlimbs or of two or more muscles within a single leg are indicated. Collectively the results of this investigation validate the use of these isometric measures to establish the functional impact associated with differences in patterns of MHC isoform expression and morphology across a broad physiological range, but also point to some limitations associated with their application.

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[2]CHAPTER 3: Effects of long-term creatine feeding and running on isometric functional measures and myosin heavy chain content of rat skeletal muscles

3.1 INTRODUCTION

Mammalian skeletal muscle fibres possess the capacity to adjust to their molecular, functional, and metabolic phenotypic properties in response to altered functional demands. Exercise training and CLFS, for example, induce the transformation of fast-twitch fibres into slower contracting and more oxidative phenotypes in rodent muscles (Pette 2002; Ren et al. 1995). However, several physiological stimuli seem to be involved in regulating these adaptive changes (Pette 2002). Green et al. (1992) suggested that the intracellular energy state of muscle fibres might influence patterns of MHC isoform expression. They reported that the intracellular phosphorylation potential (IPP), defined as the ATP/ADP_{free} ratio, was promptly diminished after the onset of CLFS, remained lower during prolonged stimulation and preceded fibre-type transitions from fast to slow. Thus, it was suggested that a lower IPP might be responsible for regulating a significant component of activity induced fast-to-slow fibre type transitions.

Conjard et al. (1998) found that in normal and chronically stimulated rodent muscles, PCr and the ATP/ADP_{free} ratio were positively correlated with the MHC-based fibre types. The relationship between MHC isoform expression and the ATP/ADP_{free} ratio suggests that a persistent change in the IPP may act as an important physiological signal, contributing to fibre type transitions. Consistent with this hypothesis, several studies have shown that when rats are fed the creatine (Cr) antagonist β-guanidinopropionic acid (β-GPA), they possess lower intramuscular concentrations of ATP and PCr, a lower ATP/ADP_{free} ratio, and associated fast-to-slow fibre-type transitions (Freyssenet et al. 1995; Petrofsky

[2] A version of this chapter has been published. Gallo M, Gordon T, Syrotuik D, Shu Y, Tyreman N, Maclean I, Kenwell Z and Putman CT. Pflügers Archiv: European Journal of Physiology. 452(6):744-55, 2006.

and Fitch 1980; Ren et al. 1995). Collectively, the results of these studies indicate that the prevailing energetic state of muscle fibres can influence changes in the MHC-based phenotype.

It has been widely documented that oral Cr supplementation increases TCr and PCr concentrations in rodent (Roy et al. 2002; Ingwall 1976; Bottnelli et al. 1994) and human (Harris et al. 1992; Hultman et al. 1996) muscles. However, it is not known if Cr feeding influences patterns of MHC isoform expression in a manner opposite to that of β-GPA. The studies of Ingwall et al. (1974 and 1976) showed that Cr selectively stimulated, by two-fold, the synthesis of actin, MHC and the cytosolic isoform of CK (MM-CK) in cultures of differentiating muscle cells. Interestingly, however, Cr did not alter the synthesis rates of soluble metabolic enzymes. Thus, it would seem that enhanced intracellular Cr combined with increased MM-CK expression has the potential to augment the efficiency and capacity for high-energy phosphate shuttling (Wallimann et al. 1998 and 1992), which could be expected to ameliorate contraction-induced reductions in the IPP and influence fibre-type transitions, especially during exercise training.

Roy et al. (2002) reported that Cr supplementation increased the cross-sectional area of histochemically identified type-II fibres in the EDL, but not in the SOL of young rats. Changes in fibre type distribution were not, however, observed in either muscle. In contrast, Brannon et al. (1997) reported that prolonged Cr feeding did not alter the cross-sectional area of type-II fibres of either the PL or SOL, but a trend toward higher IIB fibre content was noted in the PL. Collectively the results of these studies suggest a positive relationship between the IPP and the expression of fast MHC isoforms.

The PL muscle was the primary focus of this investigation because it possesses a mixed fast-twitch MHC-based phenotype and is heavily recruited during voluntary wheel running (Kariya et al. 2004). The EDL, an ankle flexor

muscle, and the MG, an ankle extensor muscle, with similar MHC fibre type compositions to the PL but comparatively low recruitment during voluntary wheel running were examined for comparison (Morse et al. 1995). Finally the SOL muscle was also included in this investigation for its slow fibre type composition. The purpose of this study was to test the hypothesis that Cr supplementation would result in a faster MHC-based phenotype in rat PL after 13 weeks of voluntary running exercise, compared with an exercise group that received no Cr. It was further hypothesised that the isometric contractile properties of the PL muscle, but not the other hindlimb muscles, would change in a corresponding manner.

3.2 MATERIALS AND METHODS

3.2.1 *Animals, experimental design and surgery*

Forty male weanling Sprague-Dawley rats (3 weeks of age) were obtained from an inbred colony maintained at the University of Alberta Animal Facility. All animals were individually housed under controlled environmental conditions (22°C and 12-h alternating light and dark cycles), and received a high protein rat chow and their respective drinking solutions ad libitum. Body mass, running distance, food and solution intake were monitored throughout the study period. All experiments were completed in accordance with the guidelines of the Canadian Council for Animal Care and received ethical approval from the University of Alberta.

The 13-week protocol was conducted in two sequential trials of 20 rats in each. Prior to the beginning of each trial, rats were categorised as either high or low runners according to a 1-week pre-selection protocol on activity wheels as described by Morse et al. 1995. Briefly, the rats were ranked according to their weekly total distance-ran. High runners were selected as the 10 rats in each trial that ran the longest total distances (total distance ran = number of revolutions x

1.1m wheel circumference) and were randomly assigned to one of the two running groups (see below). The remainder were classified as low runners and assigned to one of the two sedentary groups (see below). The total number of wheel-revolutions for each 12hr period and the daily average distance (in meters) were recorded. Each wheel revolution was detected with the use of two optical sensors, which were mounted 180° out of phase with one another on a custom-made printed circuit board with signal conditioning electronics that also included a type-D flip-flop. This permitted counting of full wheel revolutions and eliminated inclusion of partial revolutions in the calculation of total distance ran. All wheel data was recorded with a custom written LabVIEW software on a Pentium II CPU computer.

At the beginning of each trial, the high runners were randomly assigned to either the Creatine-Run (Cre-Run) or Control-Run (Con-Run) groups. Those rats that ran the shortest distances were randomly assigned to the Creatine-Sedentary (Cre-Sed) or Control-Sedentary (Con-Sed). Assignment of rats to the running or non-running groups was completed according to Morse et al. (1995); comparisons within an activity level were emphasised. An equal number of rats were included in each of the experimental groups for each trial. The Cre groups consumed a solution of 1% (w/v) creatine (1g/100mL) in 5% (w/v) dextrose while the control (Con) groups ingested a solution of 5% (w/v) dextrose. An established voluntary exercise protocol was used by Morse et al. (1995). Running groups had access to the wheels for 12 hr/day.

Surgeries were performed under general anaesthesia (45 mg/kg sodium pentobarbital IP) according to Gallo et al. (2004; see Chapter 2.2.1). An indwelling catheter (PE 50) was inserted into the external jugular, and the trachea was cannulated in the event that mechanical ventilation was required. Additional intravenous anaesthetic (diluted 1:5) was given in a saline solution (with 5% glucose) throughout all procedures as required. Incisions were made along the dorsum of the right and left hind limbs and the ankle tendons of the

EDL, SOL, MG and PL muscles were separated and individually tied with a 2.0 silk, while the TA was denervated. A silastic nerve cuff embedded with 2 multistranded stainless steel wires (AS632) was positioned around the sciatic nerve for electrical stimulation. The 2.0 silk was then attached to a Kulite strain gauge (model KH-102) for sequential force recordings. During the isolation of each muscle tendon, extreme care was taken to maintain intact arterial supply and venous drainage. Before collecting the isometric functional measures, skin incisions were loosely sutured. Throughout the experiment, animals were in a prone position and secured with clamps at the ankle and knee joints. Core body temperature was continuously monitored with a rectal probe and maintained at 39^0C with a heating pad.

3.2.2 *Isometric functional measurements and muscle sampling*

Isometric muscle function measures were completed as previously described by Gallo et al. (2004) (see Chapter 2.2.2). Before recording the measures, the optimal resting length (L_o) required to generate maximum force was established for each muscle. Maximum twitch (TW_f: mN), tetanic (TET_f: mN) contractile force, time-to-peak tension (TTP: ms), half-rise time (½RT: ms), half-fall time (½FT: ms) and sag ratio were sequentially recorded in the right EDL, PL, SOL and MG. A new fatigue protocol was designed to investigate the fatigue index (FI: %) of each muscle after 10s and 30s of stimulation, that was modelled after one typically used in humans (McCarteney et al. 1983). Muscles were stimulated at 200Hz (300 ms pulse trains) for 30s followed by a 4min rest recovery period, and repeated twice. The fatigue index (FI) was calculated after 10s (FI_{10s}) and 30s (FI_{30s}) of stimulation, as follows: [(initial tetanic force - force at 10s or 30s) / initial tetanic force] x 100. Because the FI_{10s} and FI_{30s} were similar for all three bouts and muscles fully recovered before each subsequent bout, the data were averaged. All measurements were amplified, viewed on an oscilloscope, averaged using a PDP11-lab computer and stored on disks.

The EDL, PL, SOL and MG muscles were isolated from the right hind limb, freeze-clamped with metal clamps pre-cooled in liquid nitrogen (-196 °C) and plunged into liquid nitrogen. The same muscles were extracted from the left hind limbs, weighed, fixed in a slightly stretched position and rapidly frozen in liquid nitrogen. All muscles were stored at -80°C until analysed. The anaesthetised animals were euthanised after the collection of all muscles with an overdose of sodium pentobarbital (100mg/kg), followed by exsanguination.

3.2.3 *High-energy phosphate and total creatine measures*

The MG and PL muscles were chosen for these analyses because they are recruited during voluntary wheel running and possess a mixed fast-twitch phenotype. Frozen (-80°C) freeze-clamped muscles from the right hind limb were pulverized and extracted (100mg/mL) by homogenizing (Polytron, 20s) in ice-cold 0.4M perchloric acid (PCA) containing 0.5mM ethylene glycol-bis-β - aminoethyl ether tetraacetic acid (EGTA). After 10min on ice, the acid extract was centrifuged at 10,000xg for 2min at 4°C and an aliquot of the supernatant was neutralized with 1M K_2CO_3 to pH 6-6.5. The extract was incubated on ice for 10 min, cleared by centrifugation and the supernatant was used for high performance liquid chromatography (HPLC) separation and determination of high-energy phosphates (IMP, AMP, ADP, ATP) (Ally and Park 1992). The ADP_{free} was calculated using a value of 166 for the K_{eq} of CK reaction, and used to calculate ATP/ADP_{free}. For this calculation, post-exercised resting muscle temperature, pH, and free Mg^{2+} were assumed to be 310 Kelvin, 7.0 and 1.0 mM, respectively (Golding et al. 1995; Lawson et al. 1979).

The PL muscles from the left hind limb was chosen for this analysis because it was the primary focus of our study and can be reliably considered representative of Cr loading in all muscles studied (Brannon et al. 1997; Hultman et al. 1996). Freeze-dried left PL muscles were pulverized and homogenized in ice-cold 0.5M PCA containing 1mM ethylene diamine tetraacetic acid (EDTA).

After 20min on ice, the acid extract was centrifuged at 12,000xg for 5min at 4°C and an aliquot of the supernatant was neutralized with 2.2M $KHCO_3$ to pH 7.0. The extract was placed on ice for 10min and further centrifuged to remove the insoluble potassium perchlorate. The neutralized supernatant was used for biochemical determination of intramuscular TCr concentration. Subsequently, aliquots of each extract were added to 1M nitric acid boiled for 40 minutes, cooled at 4°C, spun at 12,000xg for 5 minutes, and further diluted with 1.55M NaOH/60mM Na_2PO_4 and 11.5mM picric acid. Colour was allowed to develop for 45min and samples were read at 513nm using an Ultraspec 3000

3.2.4 *Electrophoretic analyses of myosin heavy chain content*

Relative contents of the various MHC isoforms in the right leg PL muscles were analysed as previously described by Gallo et al. (2004; see Chapter 2.2.2).

3.2.5 *Statistical analyses*

Data are summarized as mean ± SEM. Differences between group means were assessed using a Two-Way Analysis of Variance (treatment-treatment interaction: creatine and running). When a significant F-ratio was found, differences were located using the LSD post-hoc analysis for planned comparisons. Differences in muscle mass, muscle mass-to-body mass ratio, IMP, ½ FT, FI_{10s} and FI_{30s} were analysed by a one-tailed Student's t-test based on establishment of *a priori* hypotheses that predicted the direction of change. Differences were considered significant at $P \leq 0.05$, but actual P-values are cited.

3.3 RESULTS

3.3.1 *Food intake, solution intake, creatine consumption and distance ran*

Daily food intake is summarized in Figure 3-1A. On average, the rats in the two running groups consumed 20-30% more than their sedentary counterparts. Similarly, as shown in Figure 3-1B, the rats in the running groups drank 25% more of their respective solutions than the sedentary groups. Consequently the average daily Cr-intake was also 25% higher in Cre-Run group (Cre-Sed: 2.4 ± 0.17 g/kg vs. Cre-Run: 3.0 ± 0.14 g/kg; $P < 0.02$). The daily distance ran varied between 3 and 7 km per day for both Cre-Run and Con-Run groups. They did not, however, differ in the averaged daily distance ran (Cre-Run: 4,629 ± 1,317.4 m and Con-Run: 5,410 ± 1,213.8 m, $P > 0.64$) or in the total distance ran throughout the study (Cre-Run: 421,208 m and Con-Run: 492,332 m, $P > 0.64$).

3.3.2 *Body mass, muscle mass and muscle-to-body mass ratio*

Body mass increased at similar rates for all treatment groups until week 9, whereupon the effect of running in reducing body mass became apparent and remained throughout the study. Final body mass at 13 weeks was as follows: Cre-Sed; 611 ± 23.0 g, Cre-Run; 527 ± 15.2, Con-Sed; 608 ± 29.7, Con-Run; 503 ± 17.0. Rats in the Cre-Run and Con-Run groups were 14% (84 g) and 17% (105 g) lighter than their sedentary counterparts, respectively ($P < 0.0002$). The mass of the PL and MG muscles ($P < 0.05$) was reduced by running, while that of the SOL and EDL muscles was not (Table 3-1). The PL was the only muscle to display differences in the muscle mass-to-body mass ratio (Table 3-1), being larger in the Con-Run group compared with the Con-Sed ($P < 0.03$).

The wet-to-dry ratio of the PL was also measured to evaluate muscle hydration status. The wet-to-dry ratio of the PL was not affected by running or Cr

treatments (Cre-Sed: 4.03 ± 0.134; Cre-Run: 4.10 ± 0.098; Con-Sed: 4.20 ± 0.141; Con-Run: 4.05 ± 0.076, $P \geq 0.33$).

3.3.3 *Isometric functional measures of force, speed and fatigability*

The TTP, ½ RT and ½ FT measures are summarized in Table 3-2; measures within the control groups were directly comparable to previous studies (Close et al. 1969; Petrofsky and Fitch 1980). Significant differences were apparent between the SOL, EDL, MG and PL for TTP ($P < 0.01$) and ½ FT ($P < 0.0003$). The ½ RT for the SOL and EDL differed from one another and the MG differed from the PL ($P < 0.00006$). Cr, voluntary running and the combination of these treatments had no effect on the speed of contraction or relaxation within muscles. The only exception occurred in the EDL, where the ½ FT of Con-Run was 25% slower than Con-Sed ($P < 0.02$).

Measures of muscle strength as reflected in TW_f and TET_f, and the ability to sustain force, as measured by the sag ratio, are summarized in Table 3-3. TET_f differed between the various hindlimb muscles ($P<0.02$). Creatine treatment increased the TET_f generated by the PL within the sedentary groups by 18% ($P<0.05$). Conversely, Cr treatment had no significant effect on TW_f and TET_f for any of the muscles in the running groups. The sag ratio differed between muscles but was not affected by any of the treatments.

Skeletal muscle fatigue indexes at 10s and 30s are known to vary inversely with the magnitude of PCr content and with oxidative metabolism, respectively (Spriet et al. 1985 and Trump et al. 1995). The fast-twitch muscles in our study were clearly more fatigable compared with the slow-twitch SOL, as reflected by the higher values of FI_{10s} and FI_{30s} (Figure 3-2). Cr improved fatigue resistance in the SOL of the sedentary groups by 14% and 20% after 10s and 30s of stimulation, respectively ($P<0.04$). The three fast-twitch muscles investigated displayed increased fatigue resistance (i.e. reduced FI_{10s}) in

response to Cr treatment. The EDL displayed 20% and 7% greater fatigue resistance after Cr feeding in the sedentary groups after 10s and 30s of stimulation, respectively ($P < 0.02$). Similar improvements in fatigue resistance were noted after Cr loading (i.e., lower FI_{10s}) in the MG ($P<0.02$). In the PL, Cr loading resulted in lower FI_{10s} only in the running group ($P<0.02$). Running alone, on the other hand, improved fatigue resistance after 10s and 30s independent of Cr feeding ($P < 0.02$).

3.3.4 *High-energy phosphates and total creatine levels*

The effects of prolonged Cr feeding and running on the concentrations high-energy phosphate compounds in the MG and PL are summarized in Table 4. Neither Cr nor running had any effects on the resting ATP/ADP_{free} ratio or high-energy phosphate levels in the MG or PL.

TCr was measured only in the PL, as it was considered representative of all muscles (Roy et al. 2002 and Adams et al. 1995) and was the primary focus of our study. Cr loading was evident by the elevation of TCr (~20-22%) in the Cre-Run group compared with the Con-Run group (Figure 3-3). Cr loading was also confirmed for all muscles in the sedentary groups by the increased fatigue resistance at 10s (i.e. lower FI_{10s}), which has previously been shown to directly result from increases in TCr and PCr stores (Hultman et al. 1996; Trump et al. 1996; Greenhaff et al. 1994).

3.3.5 *Myosin heavy chain isoform content*

The method used to isolate and quantify MHC isoforms is shown in Figure 3-4 and the corresponding data are in Table 3-5 and Figure 3-5. With the exception of the PL muscle, voluntary running did not induce fibre-type changes in the fast-twitch muscles. There was, however, a small conversion of MHCI to MHCIIa (7%) in the SOL muscle in the running Cr loaded group ($P < 0.05$) (Table

3-5 and Figure 3-4A). The PL displayed substantial MHCIIb-to-MHCIIa transitions in response to voluntary running that encompassed 6% and 12% increases in MHCIIa ($P < 0.0002$) and MHCIId/x ($P < 0.0002$) respectively, and a 19% decrease in MHCIIb ($P < 0.000001$). When Cr was superimposed on running (Cre-Run compared with the Con-Run group), the MHC-isoform transition occurred in the opposite direction; MHCIId/x → MHCIIb (($P < 0.02$). Cr feeding abolished this running-induced transition in the ($P < 0.02$). Cr feeding alone (i.e., Cre-Sed) was associated with a 12% increase in MHCIId/x ($P < 0.0001$) and a 12% decrease in MHCIIb ($P < 0.0006$), compared with the Con-Sed group (Figures 3-4D and 3-5).

Table 3-1

Muscle mass and muscle-to-body mass ratio of rats after creatine feeding and exercise training

Muscle	Diet	Activity Level	Muscle Mass (mg)	Muscle-to-Body Mass Ratio
Soleus	Creatine	Sedentary	252.0 ± 9.53	0.41 ± 0.011
		Run	258.6 ± 21.63	0.46 ± 0.030
	Control	Sedentary	275.7 ± 17.24	0.45 ± 0.019
		Run	263.9 ± 22.50	0.51 ± 0.015
Extensor digitorum longus	Creatine	Sedentary	270.0 ± 14.66	0.44 ± 0.013
		Run	233.7 ± 17.30	0.45 ± 0.034
	Control	Sedentary	296.1 ± 21.70	0.48 ± 0.036
		Run	253.1 ± 31.62	0.46 ± 0.019
Medial gastrocnemius	Creatine	Sedentary	1639.1 ± 51.11 [a]	2.70 ± 0.075
		Run	1323.3 ± 69.23	2.56 ± 0.013
	Control	Sedentary	1486.3 ± 82.00 [a]	2.45 ± 0.074
		Run	1239.1 ± 113.33	2.38 ± 0.141
Plantaris	Creatine	Sedentary	615.0 ± 26.33 [a]	1.00 ± 0.028
		Run	550.0 ± 23.77	1.02 ± 0.047
	Control	Sedentary	601.7 ± 35.10	0.99 ± 0.023 [a]
		Run	565.6 ± 41.56	1.09 ± 0.039

Data are means ± SEM. "a" indicates sedentary is different than run within a diet condition, $P < 0.05$.

Table 3-2

Contractile properties of rat muscles after creatine feeding and exercise training

Muscle	Diet	Activity Level	TTP (ms)	½ RT (ms)	½ FT (ms)
Soleus	Creatine	Sedentary	57.8 ± 3.53	34.3 ± 2.50	57.7 ± 4.85
		Run	53.2 ± 3.90	31.5 ± 2.08	50.2 ± 3.91
	Control	Sedentary	55.8 ± 2.12	32.3 ± 2.87	59.2 ± 5.92
		Run	47.2 ± 3.69	26.1 ± 2.01	51.9 ± 3.66
Extensor digitorum longus	Creatine	Sedentary	43.8 ± 1.38	23.0 ± 1.46	28.7 ± 3.14
		Run	42.7 ± 1.76	23.3 ± 1.29	27.1 ± 3.68
	Control	Sedentary	41.5 ± 1.11	23.5 ± 1.15	21.4 ± 2.11 [a]
		Run	42.2 ± 1.00	22.2 ± 1.47	29.2 ± 2.71
Medial gastrocnemius	Creatine	Sedentary	26.9 ± 0.84	16.7 ± 0.93	13.0 ± 0.51
		Run	27.5 ± 0.73	17.8 ± 1.05	14.5 ± 0.67
	Control	Sedentary	27.3 ± 0.97	17.0 ± 1.00	14.2 ± 0.66
		Run	27.6 ± 0.98	17.2 ± 1.07	14.1 ± 0.42
Plantaris	Creatine	Sedentary	30.5 ± 1.28	18.4 ± 1.39	19.0 ± 1.55
		Run	32.9 ± 1.40	19.3 ± 1.04	18.8 ± 1.62
	Control	Sedentary	29.2 ± 1.68	17.6 ± 1.25	18.9 ± 1.37
		Run	31.5 ± 1.34	18.1 ± 1.26	19.0 ± 1.39

Data are means ± SEM. TTP is time-to-peak tension. ½RT is the half-rise time. ½FT is the half-time for muscle relaxation. "a" indicates sedentary is different than run within a diet condition, $P < 0.05$. All muscles are different from each other.

Table 3-3

Isometric force production of muscles after creatine feeding and exercise training

Muscle	Diet	Activity Level	TW_f (mN)	TET_f (mN)	Sag ratio
Soleus	Creatine	Sedentary	485 ± 49.7	2754 ± 138.5 [b]	4.05 ± 0.165
		Run	450 ± 54.8	2735 ± 194.0	4.26 ± 0.205
	Control	Sedentary	603 ± 77.4	3309 ± 140.5	3.92 ± 0.159
		Run	584 ± 72.6	3025 ± 180.3	3.93 ± 0.186
Extensor digitorum longus	Creatine	Sedentary	1319 ± 112.8	3570 ± 161.5	0.98 ± 0.097
		Run	1227 ± 120.2	3313 ± 185.3	0.86 ± 0.025
	Control	Sedentary	1403 ± 182.7	3909 ± 170.0 [a]	0.80 ± 0.033
		Run	1195 ± 129.2	3241 ± 154.0	0.90 ± 0.073
Medial gastroc-nemius	Creatine	Sedentary	1426 ± 91.4	6859 ± 410.6	1.30 ± 0.085
		Run	1280 ± 129.3	6918 ± 429.5	1.40 ± 0.090
	Control	Sedentary	1386 ± 122.7	6606 ± 380.3	1.36 ± 0.120
		Run	1281 ± 109.0	6606 ± 500.7	1.33 ± 0.115
Plantaris	Creatine	Sedentary	1400 ± 131.0	6349 ± 480.0 [b]	1.22 ± 0.095
		Run	1565 ± 273.3	6258 ± 364.1	1.34 ± 0.086
	Control	Sedentary	1177 ± 141.7	5365 ± 356.4	1.28 ± 0.171
		Run	1566 ± 226.1	6025 ± 254.5	1.27 ± 0.120

Data are means ± SEM. TW_f is twitch force. TET_f is tetanic force. "a" indicates sedentary is different than run within a diet condition and "b" indicates creatine is different than control within an activity level, $P < 0.05$.

Table 3-4

Concentrations of high-energy phosphates in rat muscles after creatine feeding and exercise training

Muscle	Diet	Activity Level	IMP	AMP	ADP	ATP	ADP_{free}	ATP/ADP_{free}
Medial gastroc-nemius	Creatine	Sed	2.04 ± 0.365 [b]	0.31 ± 0.018 [a,b]	4.5 ± 0.28	21.7 ± 2.00	11.9 ± 0.80	377 ± 40.9
		Run	3.03 ± 0.572	0.40 ± 0.023	4.6 ± 0.51	22.4 ± 1.19	13.9 ± 1.39	335± 26.0
	Control	Sed	3.04 ± 0.437	0.45 ± 0.033	4.3 ± 0.22	23.8 ± 0.57	11.8 ± 0.98	365 ± 20.4
		Run	3.20 ± 0.582	0.35 ± 0.043	4.0 ± 0.64	19.5 ± 2.34	9.5 ± 2.61	322 ± 39.2
Plantaris	Creatine	Sed	0.82 ± 0.137	0.46 ± 0.022	3.9 ± 0.15 [a]	20.4 ± 0.97	12.3 ± 1.36 [a]	368 ± 46.4
		Run	0.71 ± 0.167	0.55 ± 0.053	4.6 ± 0.03	23.3 ± 1.28	19.0 ± 3.59 [b]	331 ± 34.1
	Control	Sed	0.80 ± 0.203	0.46 ± 0.013	4.1 ± 0.15	22.9 ± 0.68	11.3 ± 1.74	488 ± 72.3
		Run	0.62 ± 0.208	0.54 ± 0.043	4.4 ± 0.21	23.3 ± 1.03	11.5 ± 0.90	379 ± 30.5

Data are means ± SEM and expressed as mmol / kg of dry wgt, with the exception of the ADP_{free} and ATP/ADP_{free}. ADP_{free} is expressed as μmol / kg wet wgt. a" indicates sedentary is different than run within a diet condition ($P < 0.05$). "b" indicates creatine is different than control within an activity level ($P < 0.05$). "Sed": sedentary group.

Table 3-5

Myosin heavy chain composition of rat soleus, extensor digitorum longus and medial gastrocnemius

Muscle	Diet	Activity Level	MHCI	MHCIIa	MHCIId/x	MHCIIb
Soleus	Creatine	Sedentary	86.3 ± 2.40 [a]	13.6 ± 2.39		
		Run	79.4 ± 2.25	20.6 ± 2.26		
	Control	Sedentary	86.0 ± 2.01	14.0 ± 2.01		
		Run	80.9 ± 2.56	17.2 ± 2.97		
Extensor digitorum longus	Creatine	Sedentary	3.9 ± 1.02	9.6 ± 1.08	37.4 ± 2.00	48.4 ± 1.97
		Run	3.1 ± 0.31	12.6 ± 0.81	37.5 ± 1.28	46.9 ± 1.19
	Control	Sedentary	3.6 ± 0.50	8.3 ± 1.23	40.6 ± 1.84	49.7 ± 1.60
		Run	5.3 ± 1.18	11.1 ± 1.92	39.8 ± 1.97	47.2 ± 1.92
Medial gastrocnemius	Creatine	Sedentary	5.9 ± 0.89	7.1 ± 1.10	31.9 ± 1.99	55.4 ± 2.32
		Run	7.4 ± 0.92	10.8 ± 0.59	30.1 ± 1.15	50.4 ± 1.29
	Control	Sedentary	6.8 ± 0.60	8.4 ± 0.66	28.3 ± 1.97	55.1 ± 1.75
		Run	6.1 ± 0.60	9.2 ± 1.09	30.4 ± 2.58	51.8 ± 2.72

Data are means ± SEM. "a" indicates sedentary is different than run within a diet condition, $P < 0.05$.

Figure 3-1

Daily food intake (A), daily volume of solution consumed (B). "a" indicates Cre-Run is greater than Cre-Sed. "b" indicates Con-Run is greater than Con-Sed. "c" indicates Cre-Sed is greater than Con-Sed. Differences between group means were assessed using a Two-Way ANOVA for each time point.

Figure 3-1

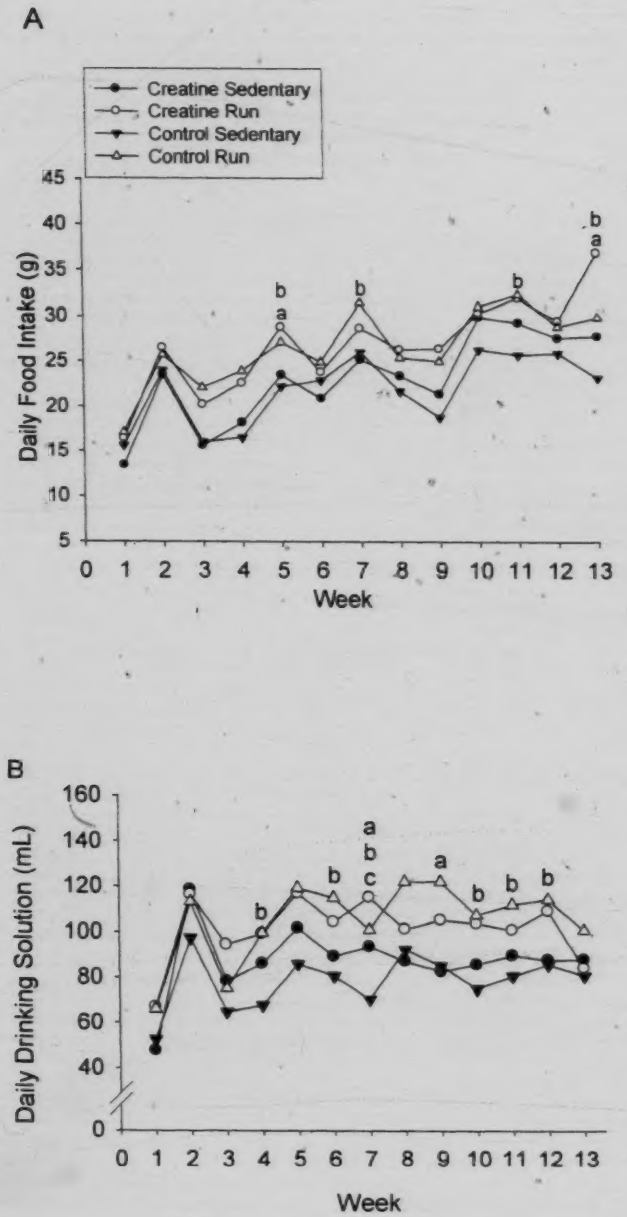

A
Creatine Sedentary
Creatine Run
Control Sedentary
Control Run
Daily Food Intake (g)
Week
B
Daily Drinking Solution (mL)
Week

Figure 3-2

Averaged fatigue index calculated after 10s (FI_{10s}) (A) and after 30s of stimulation (FI_{30s}) (B). "a" indicates Cre-Sed is different than Cre-Run. "b" indicates Con-Sed is different from Con-Run. "c" indicates Cre-Sed is different from Con-Sed. "d" indicates Cre-Run is different from Con-Run. Differences between group means were assessed using a Two-Way ANOVA.

Figure 3-2

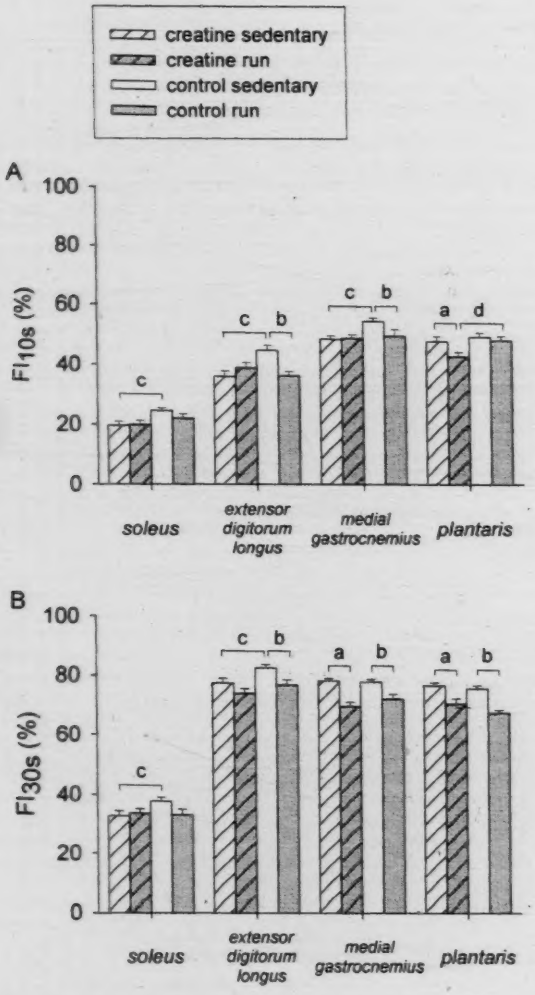

creatine sedentary
creatine run
control sedentary
control run
A
FI_{10s} (%)
100
80
60
40
20
0
c
c b
c b
a d
soleus
extensor digitorum longus
medial gastrocnemius
plantaris
B
FI_{30s} (%)
100
80
60
40
20
0
c
c b
a b
a b
soleus
extensor digitorum longus
medial gastrocnemius
plantaris

Figure 3-3

Total creatine content in the plantaris muscle. "a" indicates Cre-Sed is less than Cre-Run. "d" indicates Cre-Run is greater than Con-Run and Con-Sed. Differences between group means were assessed using a Two-Way ANOVA.

Figure 3-3

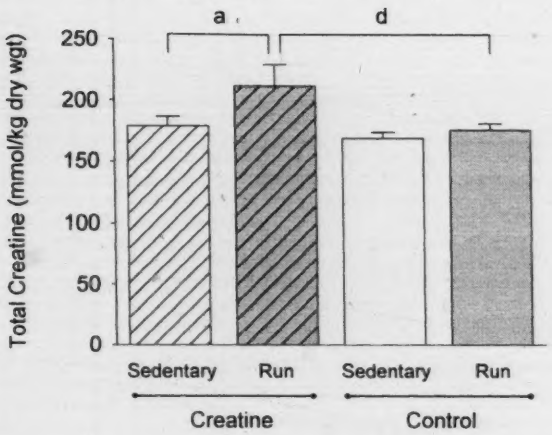

a
d
250
200
150
100
50
0
Total Creatine (mmol/kg dry wgt)
Sedentary
Run
Sedentary
Run
Creatine
Control

Figure 3-4

Electrophoretic method used to separate and quantify myosin heavy chain isoforms in the soleus (A), extensor digitorum longus (B), medial gastrocnemius (C) and plantaris (D).

Figure 3-4

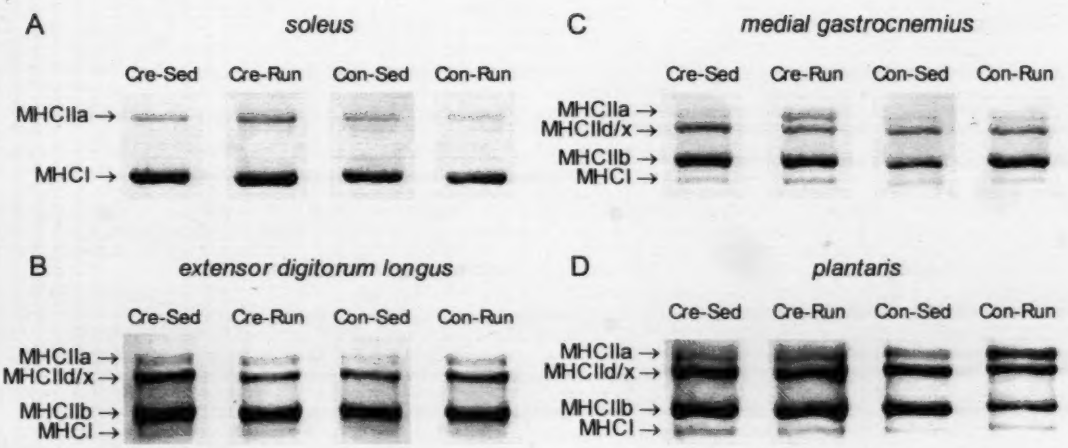
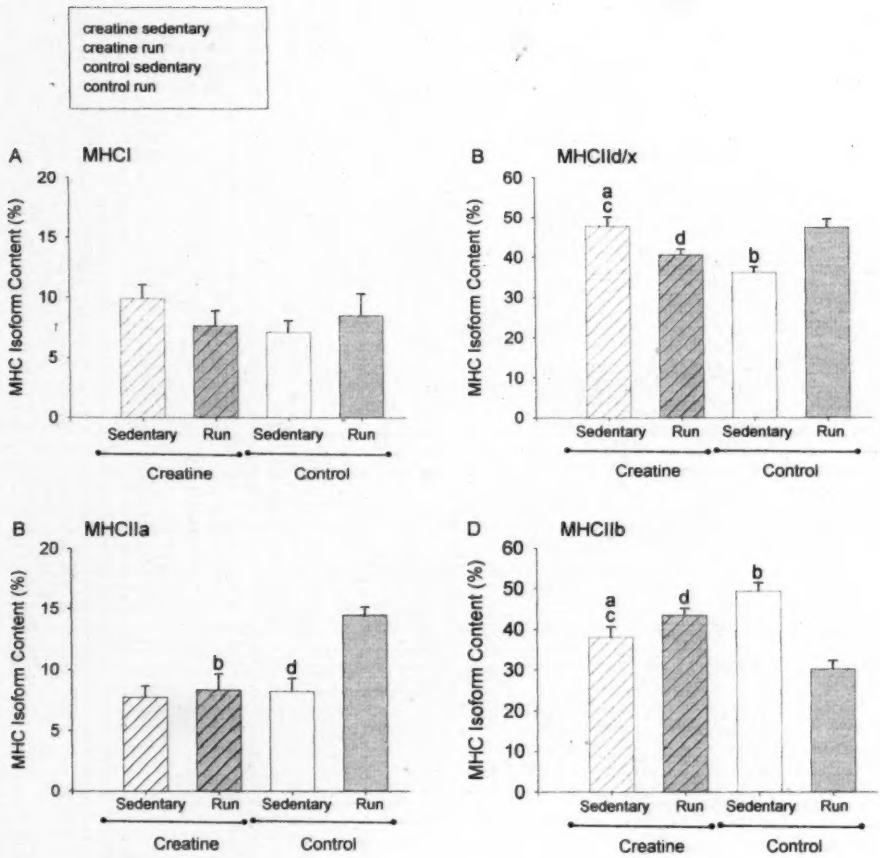

A
soleus
Cre-Sed
Cre-Run
Con-Sed
Con-Run
MHCIIa →
MHCI →
C
medial gastrocnemius
Cre-Sed
Cre-Run
Con-Sed
Con-Run
MHCIIa →
MHCIId/x →
MHCIIb →
MHCI →
B
extensor digitorum longus
Cre-Sed
Cre-Run
Con-Sed
Con-Run
MHCIIa →
MHCIId/x →
MHCIIb →
MHCI →
D
plantaris
Cre-Sed
Cre-Run
Con-Sed
Con-Run
MHCIIa →
MHCIId/x →
MHCIIb →
MHCI →

Figure 3-5

Myosin heavy chain isoform composition of plantaris muscle. MHCI content (A), MHCIIa (B), MHCIId/x (C) and MHCIIb (D). "a" indicates Cre-Sed is different than Cre-Run. "b" indicates Con-Sed is different from Con-Run. "c" indicates Cre-Sed is different from Con-Sed. "d" indicates Cre-Run is different from Con-Run. Differences between group means were assessed using a Two-Way ANOVA.

Figure 3-5


creatine sedentary
creatine run
control sedentary
control run
A
MHCI
MHC Isoform Content (%)
0
5
10
15
20
Sedentary
Run
Sedentary
Run
Creatine
Control
B
MHCIId/x
MHC Isoform Content (%)
0
10
20
30
40
50
60
a
c
d
b
Sedentary
Run
Sedentary
Run
Creatine
Control
B
MHCIIa
MHC Isoform Content (%)
0
5
10
15
20
b
d
Sedentary
Run
Sedentary
Run
Creatine
Control
D
MHCIIb
MHC Isoform Content (%)
0
10
20
30
40
50
60
a
c
d
b
Sedentary
Run
Sedentary
Run
Creatine
Control

3.4 DISCUSSION

This study employed the highest Cr dose combined with the longest longitudinal design to date. We report the novel finding that muscle Cr loading during 13 weeks of running exercise induced a faster MHC-based phenotype in the fast-twitch rat PL muscle. Surprisingly, corresponding changes in the isometric contractile properties were minimal. Despite evidence of Cr loading, either by direct biochemical measures or indirectly by greater fatigue resistance at 10s of stimulation, the selective effect of Cr supplementation on the PL appears to be related to the much greater recruitment of type IIB and IIX/D fibres during voluntary wheel-running in this muscle.

3.4.1 *Anthropometrical parameters, activity level and diet*

Voluntary running was used in our study, as opposed to treadmill running, to reduce stress-induced physiological changes (Moraska et al. 2000), which have the potential to interact with and confound physiological adaptations produced by exercise training. The young weanling rats in our study exhibited nocturnal, intermittent, high-intensity running activity similar to patterns previously reported (Morse et al. 1995; Rodnick et al. 1989), and ran at speeds that are known to elicit maximal oxygen uptake (Shepherd and Gollnick 1976). Compared with the findings of Rodnick et al. (1989), our rats could be considered "low activity" runners. Our rats, however, only had access to the running wheels for 12 hr/d compared with 24 hr access in the study of Rodnick et al. Furthermore, unlike the study of Rodnick et al., we took precautions to eliminate counting partial wheel revolutions, which are known to artificially elevate the total daily distance ran. This is because rats typically display "rocking behaviour" on activity wheels during recovery periods, leading to half revolutions being counted as full. In contrast, Morse et al. (1995) used a computer counting system similar to ours that only counted full wheel revolutions and reported similar daily running distances, in rats fed *ad libitum* (i.e., approximately 5km at 8 weeks of age).

Thus, it would seem that the running distances observed in our study could be more appropriately described as moderate. Additionally, the same average daily distance ran by very young rats at the beginning of the study may be considered as relatively high compared with the distances reported by Rodnick et al. (199) and Morse et al.(1995). Overall, the running protocol was well tolerated by all animals and Cr consumption did not interfere with fluid intake, food intake, or running behaviour.

3.4.2 *Creatine and high-energy phosphate levels*

Cr consumption in our study was 6-7 times higher than loading doses used in human studies and is the highest dose reported to date (Loike et al. 1998; Harris et al. 1992; Syrotuik et al. 2004). The higher dosage was adopted to account for the greater relative metabolic activity of rats and to maximize long-term Cr loading during the most rapid growth phase in the rat. In addition, Cr was fed in a 5% dextrose solution, to facilitate glucose-mediated-insulin release, which has been shown to further stimulate Cr transport (Steenge et al. 1998). Exercise has also been reported to stimulate glucose/Cr uptake, and probably also facilitated additional Cr entry into the exercising muscles (Robinson et al. 1999). Collectively, both glucose feeding and exercise in combination with Cr feeding would have maximized Cr uptake into the skeletal muscles. Indeed the intramuscular concentration of TCr observed in our PL muscles (Figure 3-3) was comparable to two recent studies (Brannon et al. 1997; Roy et al. 2002) and was clearly elevated in the Cre-Run group.

Increases in TCr content due to Cr feeding in the SOL, EDL and MG reported by others over 1-8 weeks (Brannon et al. 1997; McMillen et al. 2001; Roy et al. 2002) are comparable to the increments we observed within the PL, despite the fact the feeding period was longer and a much higher dose was administered. While higher doses and prolonged Cr feeding did not further enhance TCr concentration, it did maintain TCr at an elevated level

throughout the study. Based on the experiments of Loike et al. (1988), a potential maximum loading capacity seems likely, where Cr entry into the muscle may be limited by the down-regulation of Cr transporters. Indeed, Brannon et al. (1997) reported that TCr levels reached a plateau in the PL after the 14th day of Cr supplementation, and that prolonged Cr feeding, albeit at lower doses than in the present study, did not induce any further increases. The late down-regulation of Cr transport capacity may account for the absence of an elevation in TCr in the PL of the Cre-Sed group after 13-weeks. Nevertheless the apparent effect on MHC isoform switching remained (see below).

3.4.3 *Contractile properties and myosin heavy chain isoform content*

The absence of changes in the isometric twitch contractile speed (i.e., TTP, ½ RT and ½ FT) in our study was surprising, especially in the PL muscle where the significant MHCIIb to MHCIIa transitions occurred in response to daily running, which in turn was abolished by Cr feeding. Although it is not possible to discern the underlying cause(s) for the apparent disconnect between the relative MHC isoform contents and isometric measures of contraction speed within the PL, it is noteworthy that the physiological measures of contractile speed used in our study are also influenced by intracellular calcium kinetics. The absence of changes in Ca^{2+}-ATPase isoform expression in slow (SERCA1) or fast fibres (SERCA2) or in parvalbumin content could account for our observations. Alternatively, a reduction in myosin light chain (MLC) ratio of MLC_{3f} to MLC_{2f} (Brannon et al. 1997; Bottinelli 1991 and 1994) may have counteracted potential changes in the physiological function associated with changes in MHC isoform content.

Greater tetanic force in the sedentary PL muscle after Cr loading (Table 3-3), was not attributed to changes in muscle mass (Table 3-1), but was related to greater MHCIId/x content and a corresponding decrease in MHCIIb (Figure 3-5C and 3-5D). This indicates a selective effect of Cr uptake within the fast fibre

population of the PL under some conditions, not unlike the selective effects recently noted in human muscle (Syrotuik et al. 2004). The greater TET_f associated with increased MHCIId/x content corresponds to the findings of Bottinelli et al. (1991) who reported that isometric force production of individual, skinned IID(X) fibres is approximately 30% greater than that of IIB fibres. Collectively, our findings in the PL muscle show that prolonged Cr consumption can influence the MHC-based phenotype. Moreover, when contrasted with muscles of similar fibre type profile, its effects appear to be highly dependent on the initial fibre-type composition and the degree to which the muscle is recruited.

3.4.4 *Influences of the intramuscular phosphorylation potential on myosin heavy chain isoform expression*

The IPP (i.e., ATP/ADP_{free} ratio) has been proposed to be an important physiological signal that may influence MHC isoform expression (Pette 2002). Freyssenet et al. (1995), showed that 6-weeks of β-guanidinopropionic acid (β-GPA) feeding decreased the IPP in the rat EDL by reducing ATP concentration, independent of changes in ADP_{free}, which resulted in a more oxidative phenotype. Using similar experimental paradigms, Petrofsky et al. (1980) and Ren et al. (1995) reported fast-to-slower fibre type conversions in rodent muscles. More recently, Conjard et al. (1998) showed a very strong correlation between the IPP and MHC isoform expression in single fibres obtained from normal and chronically stimulated muscles. For example, thirty days of CLFS resulted in two-fold decreases in PCr and the ATP/ADP_{free} ratio, which corresponded to proportional fast-to-slow fibre type transitions. Collectively these studies demonstrate that the intramuscular cellular energetic potential can be manipulated by diet and activity, suggesting that the IPP can have a profound impact on skeletal muscle fibre phenotype.

In this study, the resting ATP/ADP_{free} ratio within MG and PL remained unaffected by Cr supplementation (Table 3-4). These findings are consistent

with a report by McMillen et al. (2001), who reported that Cr supplementation increased TCr in fast-twitch rat muscle without altering the resting ATP/ADP_{free} ratio, as expected by the equilibrium nature of CK. Although it was not possible to measure the ATP/ADP_{free} ratio during voluntary wheel running in the present study, based on previous investigations (Ingwall et al. 1974 and 1976; Bessman et al.1987; Wallimann et al. 1998), it is clear that muscles which experience prolonged increases in TCr content benefit from an enhanced rate and capacity for high-energy phosphate shuttling during activity. Consequently higher cellular ATP/ADP_{free} ratios could be maintained during repeated muscle contraction and more rapid restoration of this ratio is achieved during recovery (Eijnde et al. 2004; Greenhaff et al. 1994). Thus, it seems reasonable that a greater average IPP in the PL of the Cre-Run group contributed to the maintenance of a faster MHC phenotypic profile (Figure 3-4D and 3-5C and 3-5D), perhaps in a manner opposite to that reported by β-GPA.

While the molecular events that underlie our findings remain unknown, several possibilities are interesting. The existence of yet undiscovered energetically sensitive protein kinase(s), or cis-activating energetic response elements and their associated trans-activating factors are intriguing possibilities. Alternatively a greater IPP, during repeated muscular contractions and recovery, would be expected to enhance the uptake and sequestration of cytosolic Ca^{2+} (Pulido et al. 1998; Steeghs et al. 1997), to preserve parvalbumin content (Carroll et al. 1999; Chin et al. 2003; Schwaller et al. 1999) and consequently reduce the activation of calcineurin, a calcium-dependent protein phosphatase, which is known to stimulate slow fibre-specific gene promoters (Chin et al. 2003; Naya et al. 2000).

3.5 REFERENCES FOR CHAPTER 3

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[3]CHAPTER 4: Adaptive responses to creatine loading and exercise in fast-twitch rat skeletal muscle

4.1 INTRODUCTION

Exercise training leads to structural, contractile, and metabolic changes in rodent skeletal muscles that enhance fatigue resistance and improve efficiency of muscle contraction. Importantly, these molecular and physiological adaptations encompass the transformation of contractile proteins from fast to slower isoforms (Allen et al. 2001; Demirel et al. 1999; Kariya et al. 2004; Pette and Staron 2000; Sullivan et al. 1995) and increased oxidative capacity (Green et al. 1983; Kariya et al. 2004; Lambert and Noakes 1990; Rodnick et al. 1989). Endurance exercise training of rodent muscles has been shown to induce MHC based fibre type transitions typified by increases in type I and IIA fibres and corresponding decreases in the fastest type IID/X and IIB fibres, which appear to occur through the gradual replacement of one MHC-isoform with its slower next-nearest neighbor: MHCIIb → MHCIId/(x) → MHCIIa → MHCI (Fitzsimons et al. 1995; Green et al. 1983; Green et al. 1984; Pette 2002; Pette and Staron 1997; Staron and Pette 1993). Exercise-induced fast-to-slow fibre type transitions in rat are also accompanied by a shift from being primarily reliant on glycogenolytic ATP production to aerobic pathways of energy production (Green et al. 1983; Green et al. 1984; Lambert and Noakes 1990).

Endurance training also induces changes in the expression levels of intracellular proteins that regulate Ca^{2+} kinetics, and thus correspondingly alters the excitation-contraction-relaxation cycle. Green et al. (1984) reported substantial reductions in the content of the Ca^{2+} buffering protein, parvalbumin, in rat fast-twitch muscle that paralleled the transformation of muscle fibres from fast-fatigable to a slow-oxidative phenotype after 15 weeks of endurance training.

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Using another model of endurance training (CLFS), Pette and colleagues (Carroll et al. 1999; Gundersen et al. 1988; Huber and Pette 1996; Klug et al. 1983; Leberer and Pette 1986) reported similar reductions in parvalbumin content in several rodent models that also corresponded to fast-to-slow fibre type transitions and were found to correlate with the prolongation of muscle fibre excitation-contraction-relaxation cycle (Carroll et al. 1999; Schwaller et al. 1999). These (Carroll et al. 1999; Green et al. 1984; Gundersen et al. 1988; Huber and Pette 1996; Klug et al. 1983; Leberer et al. 1986) and other related studies (Hämäläinen et al. 1997; Hämäläinen and Pette 2001; Klug et al. 1983; Leberer et al. 1989 and 1987) also consistently showed activity-induced decreases in the fast Ca^{2+} ATPase isoform, SERCA1, content and increases in the slow isoform, SERCA2, content within rodent fast-twitch muscles. Furthermore, adaptive changes in Ca^{2+} regulation appear to be associated with muscle energy status in vivo (Chin et al. 1995; Duke and Steele 1999) as the depletion of PCr reportedly contributes to impaired Ca^{2+} regulation in muscle fibres (Duke and Steele 1999).

Recently, we (Gallo et al. 1996 and also see Chapter 3.3) showed that muscle Cr loading of rat PL enhanced the cellular energy status, which attenuated running-induced transition of MHC protein isoforms (as determined by SDS-PAGE) from fast MHCIIb to the slower MHCIIa while increasing fatigue resistance. Thus the question remains as to whether enhancing the cellular energy status by Cr loading also attenuates fast-to-slow fibre type transitions and prevents the related shift toward an oxidative phenotype while also attenuating related changes in the pattern of Ca^{2+} regulatory protein expression. The purpose of this study was to test the hypothesis that enhancing the cellular energy status of the PL by chronic Cr feeding would allow this fast-twitch muscle to retain its fast-glycolytic phenotype after prolonged voluntary running, and also maintain high levels of parvalbumin and fast SERCA1, and low levels of slow SERCA2.

4.2 MATERIALS AND METHODS

4.2.1 *Animals, experimental design and muscle sampling*

Forty male weanling Sprague-Dawley rats (3 weeks of age), which were part of a previous related study (see Chapter 3.2.1) were examined in the present study. All experiments were completed in accordance with the guidelines of the Canadian Council for Animal Care and received ethical approval from the University of Alberta Health Sciences Animal Welfare and Policy Committee. Animals were individually housed under controlled environmental conditions (22°C and 12-h alternating light and dark cycles), and ingested high protein rat chow and their assigned drinking solutions ad libitum. Body mass, food and solution intake were monitored throughout the study and are reported elsewhere (see Chapter 3.3.1 and 3.3.2). Within the run groups, TCr content of the PL muscles was elevated by 22% after 13 weeks of consuming a solution of 1% (w/v) Cr and 5% (w/v) dextrose ad libitum also as previously reported (see Chapter 3.3.4).

A 13-week endurance running protocol on activity wheels according to a procedure described by Morse et al. (1995) and Gallo et al. (1996) (also see Chapter 3.2.1). Animals were randomly assigned to one of the following 4 groups (n=10 per group): Cre-Sed - creatine and sedentary; Cre-Run - creatine and voluntary running; Con-Sed - control and sedentary; Con-Run - control and voluntary running. The running groups had access to the wheels for 12 hr/day. The Cre groups were fed a 1% Cr solution in 5% dextrose while the control (Con) groups consumed a solution of 5% dextrose.

Upon completion of the study, muscles were collected under heavy anesthesia (45 mg/kg sodium pentobarbital IP) as described in Chapter 2.2.1. PL muscles that were used for immunohistochemical analyses were isolated from the left hindlimb, weighed, fixed in a slightly stretched position, and frozen in

melting isopentane (-156°C). PL muscles from the right hindlimbs were freeze-clamped using clamps that were prechilled in liquid N_2, stored in liquid N_2 and subsequently used for biochemical analyses. Animals were euthanized with an overdose of sodium pentobarbital (100mg/kg), followed by exsanguination.

4.2.2 *Immunohistochemical staining and analyses of myofibres with antibodies against myosin heavy chain isoforms*

Monoclonal antibodies directed against adult MHC isoforms (Schiaffino et al. 1988; Schiaffino et al. 1989) were harvested from hybridoma cell lines obtained from the American Type Culture Collection: BA-D5 (IgG, anti-MHCI), SC-71 (IgG, anti-MHCIIa), BF-F3 (IgM, anti-MHCIIb); BF-35 (IgG, not MHCIId/x). Biotinylated horse-anti-mouse-IgG (rat-absorbed, affinity-purified), biotinylated horse-anti-goat-IgG and biotinylated goat-anti-mouse-IgM were obtained from Vector Laboratories. Non-specific control mouse-IgG was obtained from Santa Cruz Biochemicals.

PL muscles were mounted in embedding medium and 10μm-thick frozen sections were collected from the mid point of each muscle at -20°C. Immunocytochemical staining was completed according to Putman et al. (2003). Sections were air-dried, washed in phosphate-buffered saline (PBS) with 0.1% Tween-20 (PBS-T), with PBS, and then incubated for 15min in 3% H_2O_2 in methanol. Serial sections stained for MHCI, MHCIIa, and not MHCIId/x were incubated at room temperature for 1hr in a blocking solution (BS-1: 1% bovine serum albumin, 10% horse serum in PBS-T, pH 7.4) containing Avidin-D Blocking Reagent; on sections stained for MHCIIb goat serum was substituted for horse serum (BS-2). Sections were incubated overnight at 4°C with the primary antibody diluted in its corresponding blocking solution that contained a Biotin Blocking Reagent. Antibodies were diluted as follows: BA-D5, 1:400; SC-71, 1:100; BF-35, 1:10,000; BF-F3, 1:400. Sections were washed as before and incubated for 1 hour with biotinylated horse-anti-mouse-IgG (1:200) (MHCI,

MHCIIa, and not MHCIId/x) or biotinylated goat-anti-mouse-IgM (1:400) (MHCIIb). Sections were washed, incubated with Vectastain ABC Reagent (i.e., avidin-biotin Horse Radish Peroxidase (HRP) complex) and immunoreactivity was developed by incubating with a solution containing diaminobenzidine, H_2O_2, and $NiCl_2$ in 50 mM Tris-HCl (pH 7.5). Control samples were run in parallel in which the primary IgM antibody was omitted, or a non-specific mouse-IgG antibody was substituted. All sections were subsequently dehydrated, cleared, and mounted in Entellan.

All semi-quantitative analyses were completed with a Leitz Diaplan microscope fitted with a Pro Series High Performance CCD camera and a custom designed analytical imaging program (Putman et al. 2000). Muscle fibres stained for the various MHC isoforms were examined from three distinct cross-sectional areas of the PL muscle (i.e., deep, middle, and superficial) for each of the Cr-Sed (total fibres: 185 ± 18 fibres/muscle), Cre-Run (total fibres: 210 ± 16 fibres/muscle), Con-Sed (total fibres: 190 ± 14 fibres/muscle) and Con-Run groups (total fibres: 198 ± 19 fibres/muscle). A total of 7,830 fibres were examined for fibre type distribution analyses. Fibre area analyses were performed on the same fibres. Type-I, -IIA, and -IIB fibres were identified by positive staining of fibres and type-IID/X fibres were identified by the absence of staining with clone BF-35, as well as all other antibodies.

4.2.3 *Western blot analyses of parvalbumin and calcium ATPases*

Frozen PL muscles were pulverized under liquid N_2. An aliquot of muscle powder was diluted in 1:4 in a buffer (20mM Tris-HCl, 300mM sucrose, 0.2mM PMSF, pH 7.4) and homogenized using a glass homogenizer. Samples were stirred on ice for 20 min and centrifuged at 1000 x g for 10 min at 4^0 C. The supernatant was collected and stored at -20^0 C. Protein concentrations were determined according to Bradford et al. (1976). Samples were diluted to a final concentration of 1 μg/μl with the homogenization buffer containing 0.1% (wt/vol)

bromophenol blue and heated for 10 min at 65^0 C. Electrophoresis was performed on a 1.5mm-thick 15% (wt/vol) polyacrylamide mini-gel (3.5% stacking gel) at 25-40mA for 2hrs. Equal amounts of protein were loaded (10 μg/lane). Proteins were electrotransferred (wet) onto a PVDF membrane (62) and stained with Ponceau-S as a second confirmation of equal loading between lanes. Membranes were destained, blocked in a buffer containing skim milk powder (5% wt/vol), PBS-T (0.1%, vol/vol) for 1 hr. Membranes were then incubated for 1 hr with rabbit polyclonal anti-parvalbumin (0.1 ug/mL in the blocking solution, IgG). Membranes were then washed and incubated for 1 hr with anti-rabbit IgG-horseradish peroxidase (HRP) (1:3,000 in the blocking solution) and washed several times. Immunoreactivity was visualized with Amersham Biosciences Detection Reagents and corresponded to a molecular weight of 12 kDa, as determined by comparison with standard molecular weight markers (Precision Plus Protein Standards). All samples were evaluated in duplicate. Immunoblots were evaluated by integrating densitometry using GeneSnap and GeneTools. Membranes were reprobed with monoclonal anti-α-actinin (1:500 in blocking solution) (clone EA-53), which served as the internal control, and further confirmed equal loading between lanes. Immunoreactivity for α-actinin (100 kDa) was visualized as described above, after incubation with anti-mouse IgG (1:2,000 in blocking solution).

Frozen PL muscle samples were minced and homogenized using a glass homogenizer in a buffer (diluted 1:4) containing 50mM Tris-HCl, 1mM Na_2EDTA, 0.1% Triton X-100, and 5 mg/mL of protease inhibitor (pH 7.6). Homogenates were stirred on ice for 20 min and centrifuged at 1000 x g for 10 min at 4^0 C. The supernatant was collected and stored at -20^0 C. Protein concentrations were determined as before. Samples were further diluted as before and heated for 3 min at 85^0 C. Equal amounts of protein were loaded (50 μg/lane for SERCA1 and 80 μg/lane for SERCA2). Electrophoresis was performed on 1.5mm-thick 7% (wt/vol) polyacrylamide mini-gels (3.5% stacking gel) for 30 min at 60 V and subsequently for 90min at 140 V. Separated proteins were transferred to a

PVDF membrane (Towbin et al. 1979). Equal loading was confirmed using Ponceau-S as described above. Membranes probed for Ca^{2+} ATPase isoforms were blocked in a buffer containing skim milk powder (2.5% wt/vol) and BSA (1% wt/vol) in PBS-T (0.1%) (pH 7.4) followed by incubation with monoclonal anti-SERCA1 (1;5,000 in blocking solution) or monoclonal anti-SERCA2 (1;4,000) overnight at 4 ^{0}C. Membranes probed for SERCA1 were washed and incubated with anti-mouse IgG-HRP (1:3,000 in blocking solution) for 1 hr, washed again and developed as described above. Membranes probed for SERCA2 were incubated with biotinylated anti-mouse IgG (1:400 blocking solution) for 1 hr and incubated again for 1 hr with peroxidase-labeled streptavidin (1:500 in blocking soulution), washed and developed as before. Immunoreactivity for both Ca^{2+} ATPase isoforms corresponded to a molecular weight of 110 kDa. All samples were evaluated in duplicate as described above. Membranes were reprobed with polyclonal anti-β-actin (1:2000), which served as the internal control, and further confirmed equal loading. Immunoreactivity of β-actin was visualized as described above, after incubation with a biotinylated anti-rabbit IgG (1:2,000 in blocking solution) and peroxidase-labeled streptavidin (1:500 in blocking solution).

4.2.4 *Metabolic enzyme analyses*

For measurements of citrate synthase (CS, EC 4.1.3.7), 3-hydroxyacyl-CoA dehydrogenase (HADH, EC 1.1.1.35), glyceraldehyde phosphate dehydrogenase (GAPDH, EC 1.2.1.12), and phosphofructokinase (PFK, EC 2.7.1.11), muscles were extracted in a high-salt medium containing 5 mM EDTA, 100 mM sodium potassium phosphate buffer (pH 7.2) (Reichmann et al. 1983), with the addition of 0.1% (v/v) Triton X-100, in order to ensure complete extraction of soluble and structure-bound activities. To stabilize GAPDH, dithithreitol (DTT) was added to an aliquot of the supernatant fraction yielding a 2 mM final concentration. Similarly, to stabilize PFK, fructose 1, 6-bisphosphate and DTT were added to an aliquot of the supernatant fraction yielding final

concentrations of 1mM and 2mM, respectively. CS activity was subsequently measured at 30°C (Srere 1969). HADH activity was also determined at 30°C according to Bass (1969). PFK and GAPDH activities were immediately measured after homogenization at 30°C (Bass 1969).

4.2.5 *Statistical analyses*

Data are summarized as mean ± SEM. Differences between group means were assessed using a Two-Way Analysis of Variance (treatment-treatment interaction: creatine and running). When a significant F-ratio was found, differences were located using the LSD post-hoc analysis for planned comparisons. Differences were considered significant at $P < 0.05$, but actual P-values are cited.

4.3 RESULTS

4.3.1 *Fibre type transitions*

Fibre type transitions of the PL muscle were assessed by semi-quantitative immunohistochemical analyses on serial sections (Figure 4-1) in the deep, middle and superficial regions of each group, to ensure representative sampling. As shown in Figure 4-2, running increased the proportion of fibres expressing MHCIIa in both Cr (by 9%, $P < 0.04$) and Con (by 26%, $P < 0.0003$) groups; however, the increase was substantially lower in the Cr group ($P < 0.02$), indicating that the running-induced fibre type transitions were attenuated in the presence of Cr loading. This was reinforced by the finding that the proportion of fibres expressing MHCIIb decreased by 15% ($P < 0.004$) in the Con-Run group compared with the Cr-Run group, which did not differ from its sedentary control. The proportion of fibres expressing MHCIId/x was decreased to the same extent (by approximately 11%) in both the Cr-Run ($P < 0.03$) and Con-Run ($P < 0.05$)

compared to their corresponding sedentary controls. No changes were observed in the percentage of fibres expressing MHCI.

When individual pure and hybrid fibre types were examined, a similar pattern emerged (Figure 4-3). Running increased type IIA fibres by 24% (Figure 4-3C, $P < 0.002$) while decreasing type IID(X) and type IIB fibres (Figure 4-3E and 4-3G) by 15 % and 10 %, respectively ($P < 0.02$). However, when Cr loading was combined with running, there was no increase in type IIA fibres (Figure 4-3C), and no decrease in type IIB fibres (Figure 4-3G).

4.3.2 *Cross-sectional areas of fibres*

Figure 4-4 summarizes the cross-sectional area (CSA) of all pure and hybrid fibre types. Running alone was associated with an increase in the CSA of all pure fibre types (Con-Sed vs. Con-Run) and hybrid type IIA/IID(X). The effects of Cr varied in response to the activity conditions. In the sedentary groups, Cr loading corresponded to increases in the CSA of type I, I/IIA, IID(X) and IIB fibres by 22%, 39%, 17%, 21%, respectively ($P < 0.002$). In the running groups, however, this trend appeared to be reversed. Cr loading combined with running was associated with decreases in the CSA of type IIA/IID(X), IID(X) and IIB fibres by 29%, 17% and 9%, respectively ($P < 0.002$). This resulted from a more advanced fibre type transition from IIB to IIA in the Con-Run (Figure 4-5A) group compared to Cre-Run (Figure 4-5B) group.

4.3.3 *Parvalbumin and calcium ATPase content*

Figure 4-6A illustrates the immunoblot method used to quantify parvalbumin protein content. Parvalbumin content was highest in Con-Sed compared to all other groups (Figure 4-6B, $P < 0.03$). Running resulted in approximately 50% decrease (Con-Run vs. Con-Sed, $P < 0.03$) in parvalbumin content, which corresponded to the fast-to-slower fibre type transitions observed within the subpopulations of fast-twitch fibre types. Surprisingly, Cr feeding alone

resulted in a 70% decrease in parvalbumin content (Con-Sed vs. Cre-Sed, $P < 0.0004$), while Cr loading plus running resulted in a similarly low level of parvalbumin, which was 17% of that found in the Con-Run group.

The immunoblot methods used to compare the relative abundance of the Ca^{2+} ATPase isoforms in the PL are illustrated in Figures 4-7A and 4-8A. No significant differences were observed in the fast Ca^{2+} ATPase isoform, SERCA1, content (Figure 4-7B). As expected, running alone resulted in a 43% increase in the slow Ca^{2+} ATPase SERCA2 (Con-Sed vs. Con-Run, $P < 0.005$). Surprisingly, however, SERCA2, content was elevated by 25% (Con-Sed vs. Cre-Sed) in response to Cr loading alone (Figure 4-8B, $P < 0.03$).

4.3.4 Reference *enzyme activities*

All enzyme activities are summarized in Figure 4-9. Enzyme activities in the PL are within ranges previously reported (Bamford et al. 2003; Putman et al. 2003). As expected, running alone increased the CS (Figure 4-9A, $P < 0.00007$) and HADH (Figure 4-9B, $P < 0.05$) activities by 41% and 20%, respectively. In contrast, when Cr loading was combined with running, increases in the activities of CS and HADH did not occur (Figure 4-9A and 4-9B). No significant differences were observed in the activities of the glycolytic reference enzymes GAPDH (Figure 4-9C, $P = 0.75$) or PFK (Figure 4-9D, $P = 0.77$).

Figure 4-1

Representative photomicrographs of myosin heavy chain immunohistochemistry of the plantaris muscles for the Cre-Run (A, B, C, and D) and Con-Run (E, F, G and H) groups. A and E, immunostains of MHCI (clone BA-D5); B and F, immunostains of MHCIIa (clone SC-71); C and G, immunostains of all MHC isoforms except MHCIId/(x) (clone BF-35); D and H, immunostains of MHCIIb (clone BF-F3). Scale bar represents 100 μm.

Figure 4-1

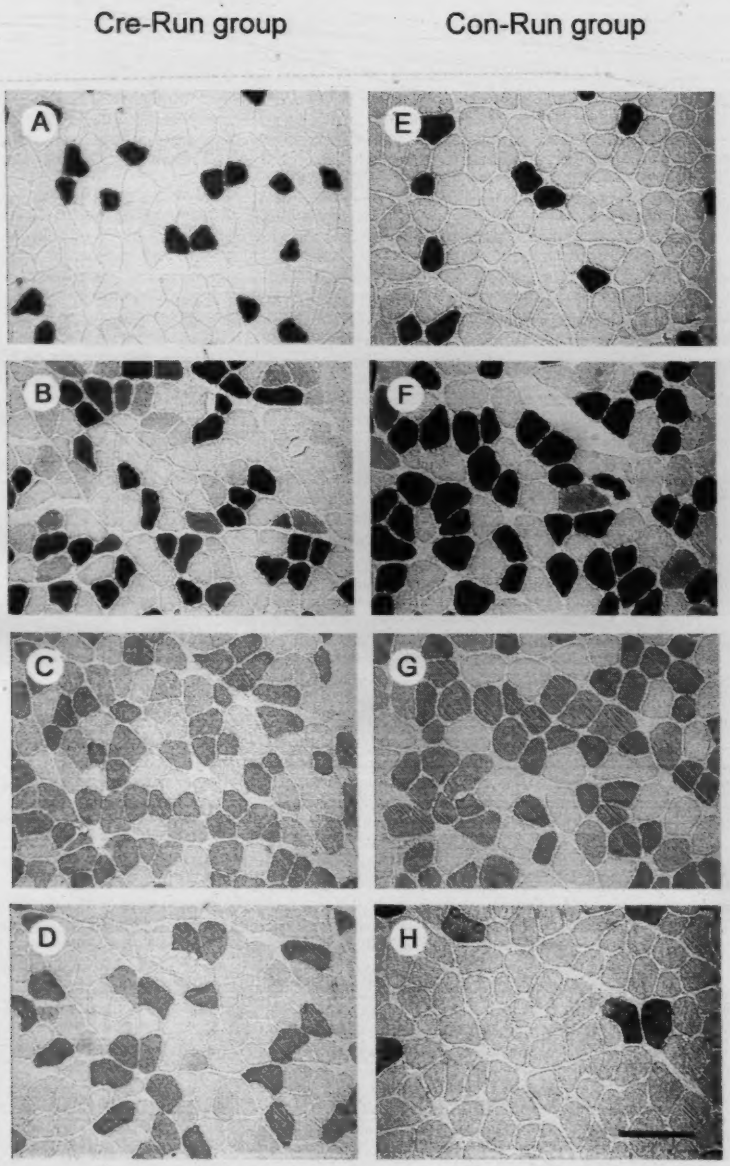


Figure 4-2

The percentage of fibres expressing a particular myosin heavy chain isoform in the plantaris muscles. "a" Cre-Run is different from Cre-Sed . "b" Con-Run is different from Con-Sed. "d" indicates Cre-Run is different from Con-Run. Differences between group means were assessed using a Two-Way ANOVA.

Figure 4-2

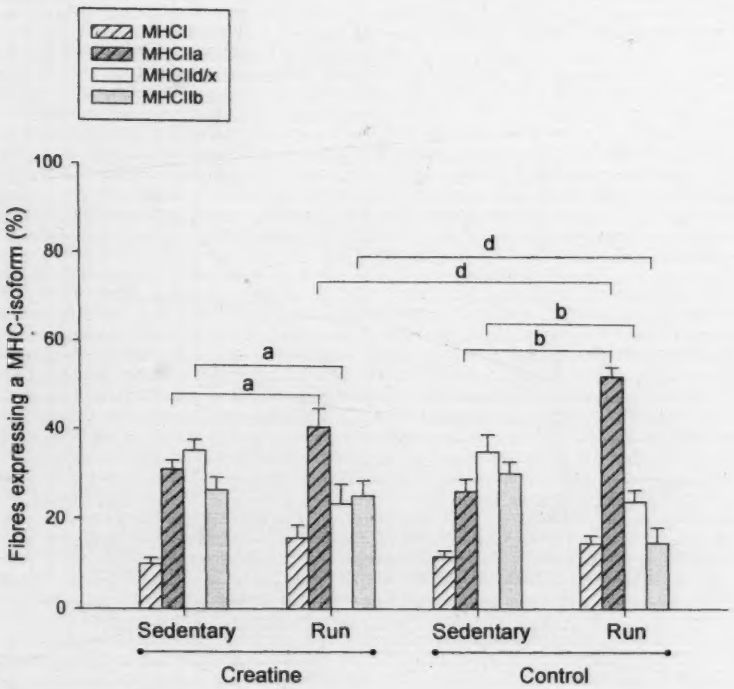

MHCI
MHCIIa
MHCIId/x
MHCIIb
Fibres expressing a MHC-isoform (%)
100
80
60
40
20
0
a
a
b
b
d
d
Sedentary
Run
Sedentary
Run
Creatine
Control

Figure 4-3

The percentage of pure and hybrid fibre types in the plantaris muscles: Type I (A), I/IIA (B), IIA (C), IIA/IID(X) (D), IID(X) (E), IID(X)/IIB (F) and IIB (G). "a" Cre-Run is different from Cre-Sed . "b" Con-Run is different from Con-Sed. "d" indicates Cre-Run is different from Con-Run. Differences between group means were assessed using a Two-Way ANOVA.

Figure 4-3

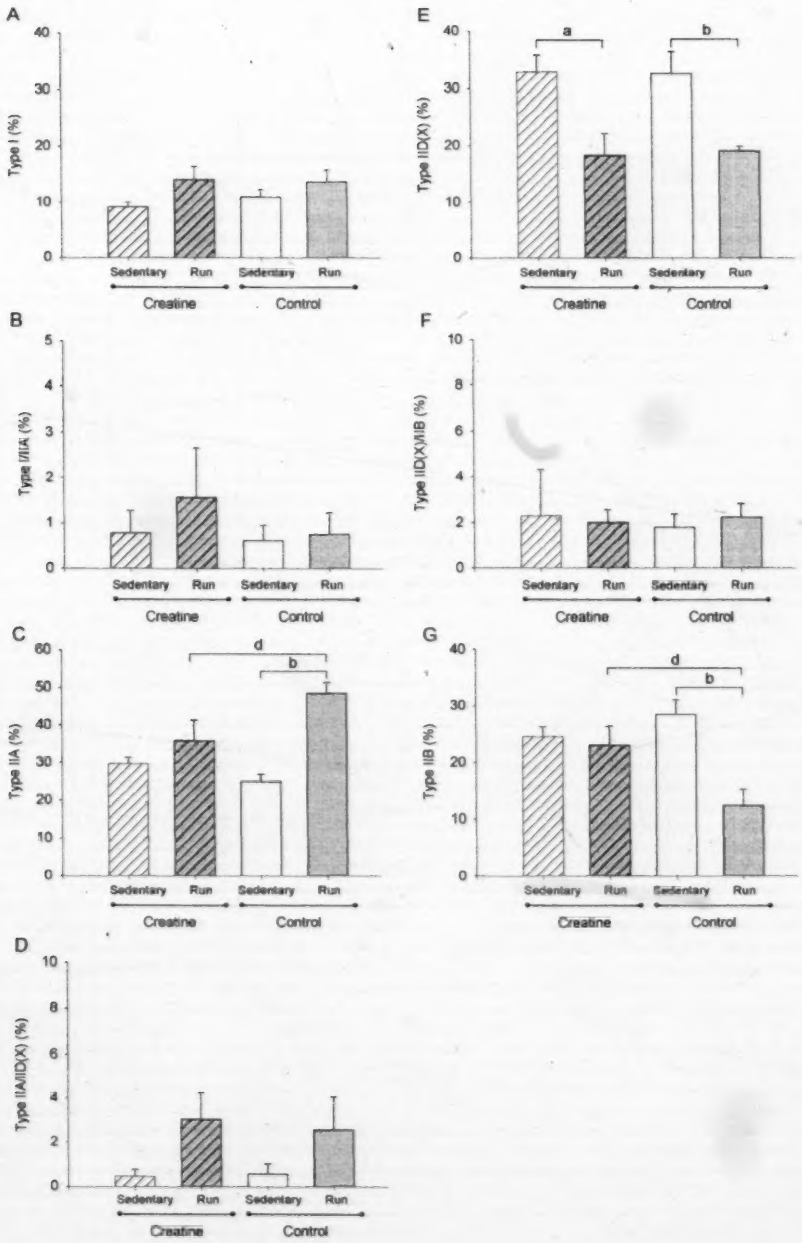

A
Type I (%)
Sedentary
Run
Creatine
Control
B
Type I/IIA (%)
C
Type IIA (%)
d
b
D
Type IIA/IID(X) (%)
E
Type IID(X) (%)
a
b
F
Type IID(X)/IIB (%)
G
Type IIB (%)

Figure 4-4

Cross-sectional area of fibre types in the plantaris muscles: Type I (A), I/IIA (B), IIA (C), IIA/IID(X) (D), IID(X) (E), IID(X)/IIB (F) and IIB (G). "a" Cre-Run is different from Cre-Sed . "b" Con-Run is different from Con-Sed. "c" indicates Cre-Sed is different from Con-Sed. "d" indicates Cre-Run is different from Con-Run. Differences between group means were assessed using a Two-Way ANOVA.

Figure 4-4

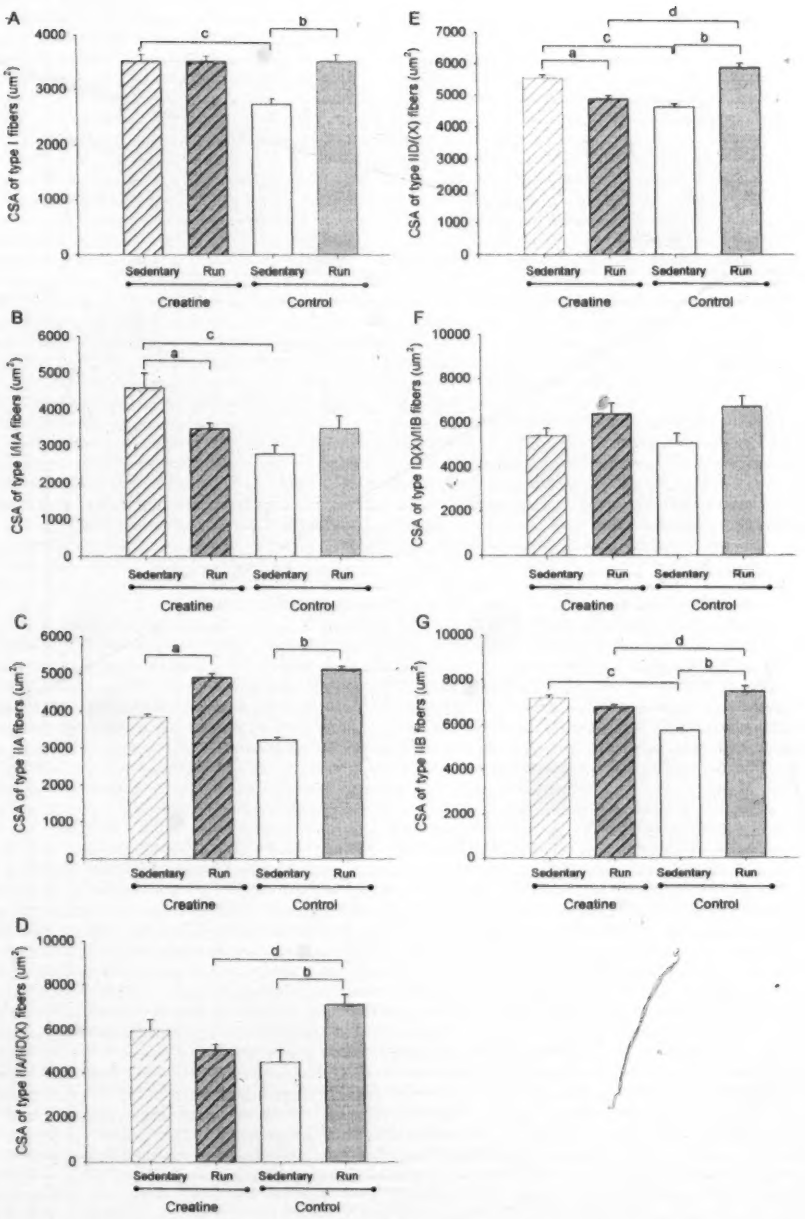

A
CSA of type I fibers (um²)
4000
3000
2000
1000
0
c
b
Sedentary
Run
Sedentary
Run
Creatine
Control
B
CSA of type I/IIA fibers (um²)
6000
5000
4000
3000
2000
1000
0
c
a
Sedentary
Run
Sedentary
Run
Creatine
Control
C
CSA of type IIA fibers (um²)
6000
5000
4000
3000
2000
1000
0
a
b
Sedentary
Run
Sedentary
Run
Creatine
Control
D
CSA of type IIA/IID(X) fibers (um²)
10000
8000
6000
4000
2000
0
d
b
Sedentary
Run
Sedentary
Run
Creatine
Control
E
CSA of type IID(X) fibers (um²)
7000
6000
5000
4000
3000
2000
1000
0
d
c
b
a
Sedentary
Run
Sedentary
Run
Creatine
Control
F
CSA of type IID(X)/IIB fibers (um²)
10000
8000
6000
4000
2000
0
Sedentary
Run
Sedentary
Run
Creatine
Control
G
CSA of type IIB fibers (um²)
10000
8000
6000
4000
2000
0
d
c
b
Sedentary
Run
Sedentary
Run
Creatine
Control

Figure 4-5

Myofibre size distribution of type IIA, IID(X) and IIB fibres of the plantaris muscles of the Con-Run (A) and Cre-Run (B) groups. The numerical values represent the mean proportion of each fibre type.

Figure 4-5

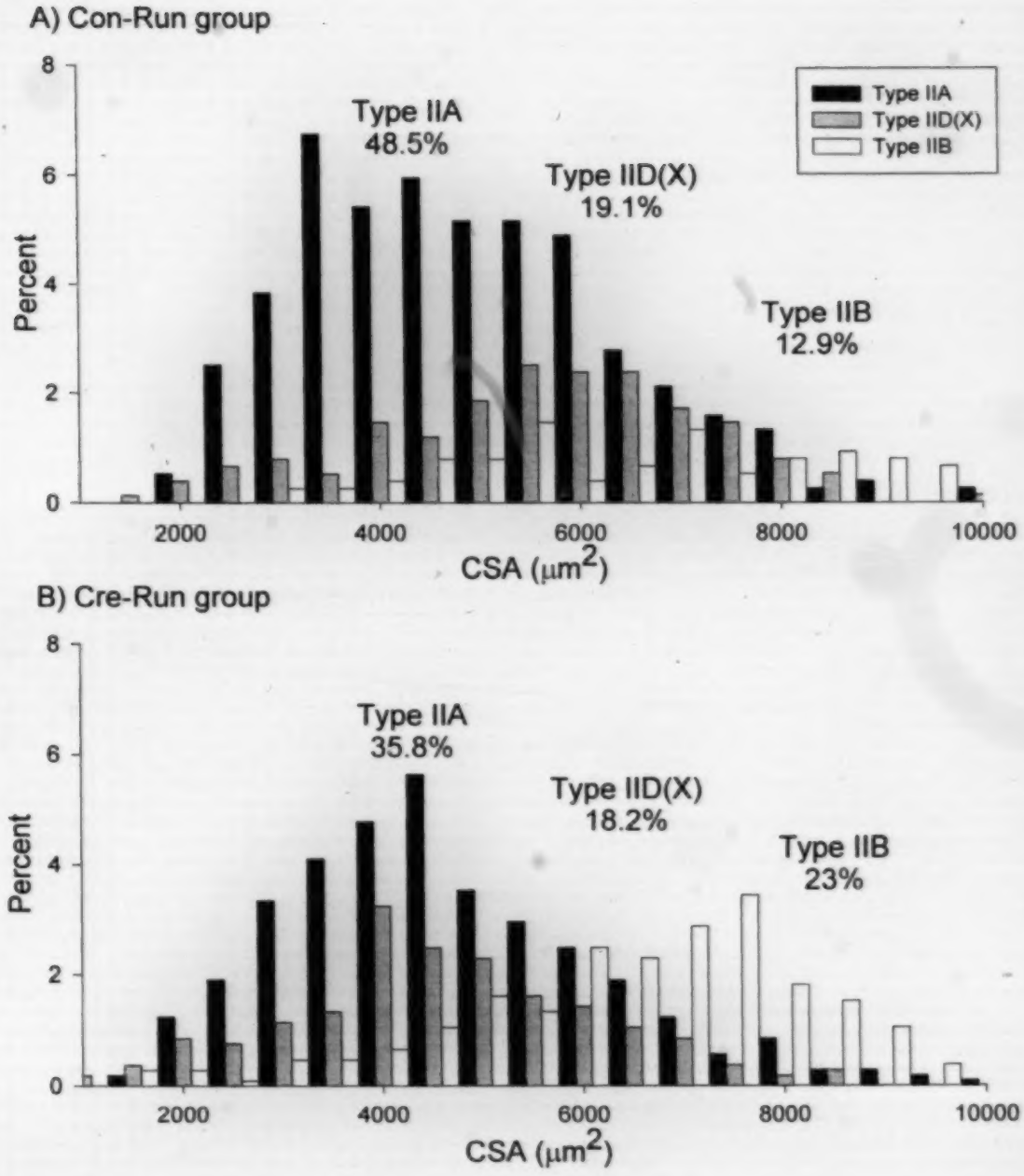
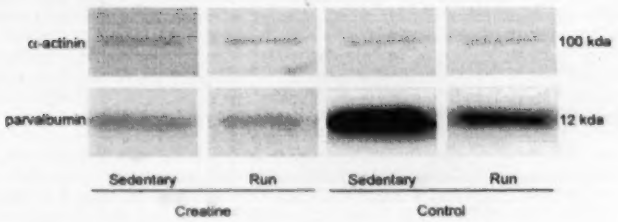

A) Con-Run group
Type IIA
Type IID(X)
Type IIB
Type IIA
48.5%
Type IID(X)
19.1%
Type IIB
12.9%
Percent
CSA (μm2)
B) Cre-Run group
Type IIA
35.8%
Type IID(X)
18.2%
Type IIB
23%
Percent
CSA (μm2)

Figure 4-6

A representative immunoblot of parvalbumin in the plantaris muscles (A). Densitometric evaluation is shown in B. Units of measure: parvalbumin / α-actinin (loading control). "a" Con-Sed is different from Con-Run. "c" indicates Cre-Sed is different from Con-Sed. "d" indicates Cre-Run is different from Con-Run. Differences between group means were assessed using a Two-Way ANOVA.

Figure 4-6

A


α-actinin
parvalbumin
100 kda
12 kda
Sedentary
Run
Sedentary
Run
Creatine
Control

B

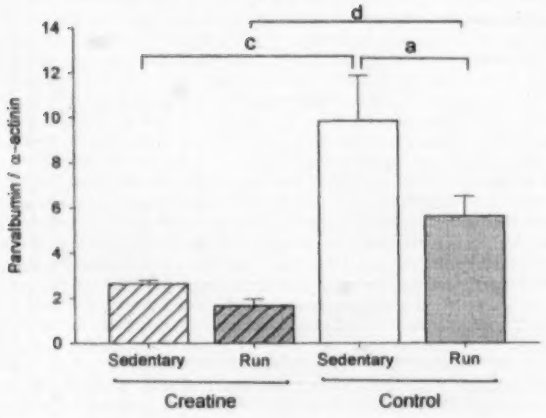

d
c
a
Parvalbumin / α-actinin
14
12
10
8
6
4
2
0
Sedentary
Run
Sedentary
Run
Creatine
Control

Figure 4-7

A representative immunoblot of SERCA1 in the plantaris muscles (A). Densitometric evaluation is shown in B. Units of measure: SERCA1 / β-actin (loading control). Differences between group means were assessed using a Two-Way ANOVA.

Figure 4-7

A

β-actin
42 kda
serca 1
110 kda
Sedentary
Run
Sedentary
Run
Creatine
Control

B

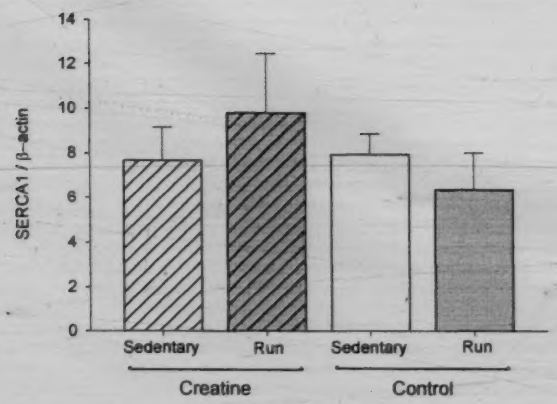
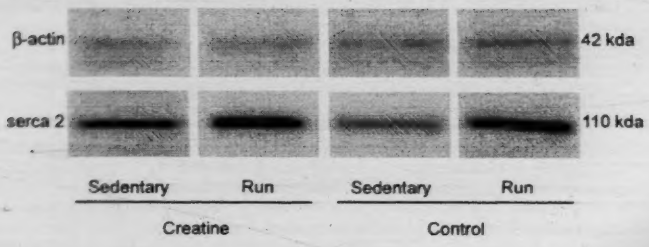

SERCA1 / β-actin
14
12
10
8
6
4
2
0
Sedentary
Run
Sedentary
Run
Creatine
Control

Figure 4-8

A representative immunoblot of SERCA2 in the plantaris muscles (A). Densitometric evaluation is shown in B. Units of measure: SERCA2 / β-actin (loading control). "a" indicates Con-Sed is different from Con-Run. "c" indicates Cre-Sed is different from Con-Sed. "d" indicates Cre-Run is different from Con-Run. Differences between group means were assessed using a Two-Way ANOVA.

Figure 4-8

A


β-actin
42 kda
serca 2
110 kda
Sedentary
Run
Sedentary
Run
Creatine
Control

B

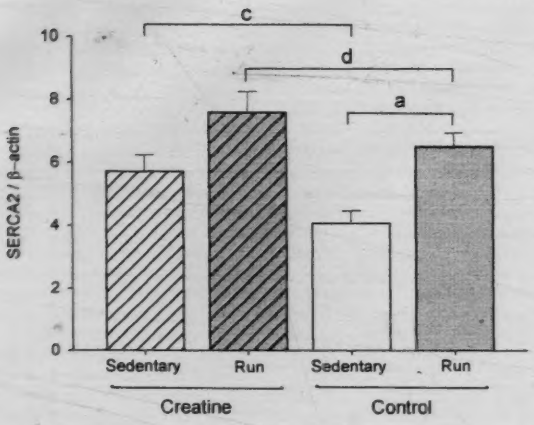
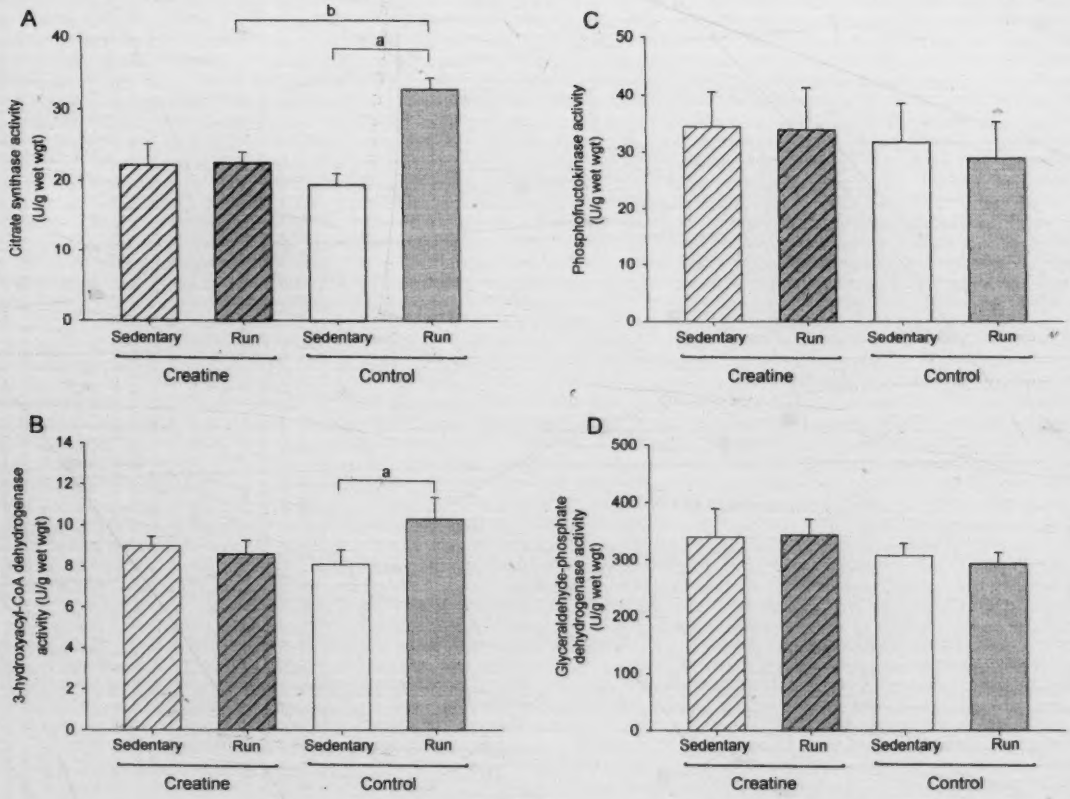

c
d
a
10
8
6
4
2
0
SERCA2 / β-actin
Sedentary
Run
Sedentary
Run
Creatine
Control

Figure 4-9

Maximum activities of citrate synthase (CS, EC 4.1.3.7; A), 3-hydroxyacyl –CoA dehydrogenase (HADH, EC 1.1.1.35; B), glyceraldehydephosphate dehydrogenase (GAPDH, EC 1.2.1.12; C) and phosphofructokinase (PFK, EC 2.7.1.11; D) in the PL muscles. Units of measure are U/g wet weight. "a" Con-Run is different from Con-Sed . "b" Cre-Run is different from Con-Run. Differences between group means were assessed using a Two-Way ANOVA.

Figure 4-9


A
Citrate synthase activity (U/g wet wgt)
40
30
20
10
0
b
a
Sedentary
Run
Sedentary
Run
Creatine
Control
C
Phosphofructokinase activity (U/g wet wgt)
50
40
30
20
10
0
Sedentary
Run
Sedentary
Run
Creatine
Control
B
3-hydroxyacyl-CoA dehydrogenase activity (U/g wet wgt)
14
12
10
8
6
4
2
0
a
Sedentary
Run
Sedentary
Run
Creatine
Control
D
Glyceraldehyde-phosphate dehydrogenase activity (U/g wet wgt)
500
400
300
200
100
0
Sedentary
Run
Sedentary
Run
Creatine
Control

4.4 DISCUSSION

Since it was first reported by Harris et al. (Harris et al. 1992), that oral Cr consumption could elevate total intramuscular Cr content within human skeletal muscle, the primary focus of subsequent studies has been on the extent to which Cr loading acutely improves fatigue resistance by enhancing the available pool of high energy phosphates (Hultman et al. 1996). In vitro (Ingwall et al. 1974) and in vivo (Brannon et al. 1997; Roy et al. 2002) studies further reveal the potential for selective myosin synthesis and muscle fibre hypertrophy with prolonged Cr loading. The present study extends the findings of those studies and of our previous work (Gallo et al. 2006 and also see Chapter 3.3), which investigated the effects of voluntary run training and Cr loading on MHC-isoform protein contents, whole muscle isometric functional properties and high-energy phosphates within the PL, to include detailed morphological analyses of muscle fibre type distribution, adaptive changes to proteins known to regulate the intracellular Ca^{2+} levels and to the metabolic phenotype. Here we report the novel findings that chronic Cr loading in the PL attenuated running-induced fast-to-slow fibre type transitions, as determined immunohistochemically, and prevented associated increases in aerobic capacity as reflected by lower activities of the reference enzymes CS and HADH. Moreover, chronic Cr loading surprisingly resulted in a dramatic decrease in the content of parvalbumin and an increase in the slow Ca^{2+} ATPase isoform, SERCA2.

4.4.1 *Fibre type transitions*

The activity pattern of rats on voluntary running wheels is characterized by many short bouts (i.e. less than 3 min) of high intensity running (i.e. 40-60 m/min) corresponding to 95-105% of maximal oxygen consumption (Rodnick et al. 1989). Our previous study showed that this training regimen induced fast-to-slow MHC-isoform transitions as determined by gel electrophoresis (see Chapter 3.3.5) that are qualitatively similar to other forms of endurance exercise training

(Pette and Staron 1997). The immunohistochemically determined fibre types in the present study are in agreement with the MHC-based isoform transitions seen in our previous study (see Chapter 3.3.5). Importantly, the immunohistochemical analyses allowed us to demarcate pure and hybrid fibre types, and to further conclude that running-induced MHC-isoform transitions were the result of fast-to-slower fibre type transitions within the fast subpopulations and not due to fibre specific hypertrophy or atrophy. Our results clearly show that high levels of intramuscular Cr prevented the reduction in IIB fibres and the rise in IIA fibres that typically occur in response to endurance running.

While the underlying mechanisms that account for our observations remain to be elucidated, there is considerable evidence that muscles which experience prolonged increases in TCr content benefit from an enhanced rate and capacity for high-energy phosphate shuttling during contractile activity (Bessman 1987; Wallimann et al. 1992 and 1998). This is supported by two studies, which showed that Cr loading more rapidly restored the ATP/ADP_{free} ratio during recovery from contractile activity (Eijnde et al. 2004; Greenhaff et al. 1994). In contrast to the fast phenotype supported by Cr loading, muscle creatine depletion by the Cr antagonist beta-guanidinopoprionic acid (β-GPA), disrupts high-energy phosphate shuttling (Freyssenet et al. 1995) and results in muscle fibre atrophy (van Deursen et al. 1994) and transformation into a slow oxidative phenotype (Freyssenet et al. 1995; Petrofsky and Fitch 1980; Ren et al. 1995; Roussel et al. 2000). The Cr feeding induced changes observed in our study, and the β–GPA induced changes reported by others, appear to act through alterations in intracellular Ca^{2+} concentration.

4.4.2 *Calcium regulatory proteins*

This study is the first to report the effects of Cr loading on the levels of protein expression involved in intracellular calcium regulation. Previous studies showed that the pattern of SERCA isoform expression is under the control of various factors including changes in contractile activity (Pette and Vrbová 1999) and active loading (Kandarian et al. 1994). Bigard and colleagues (2000) using cyclosporin A, a calcineurin inhibitor, showed that the expression of SERCA and MHC isoforms can be co-regulated by calcineurin, and that a strong co-expression of SERCA1 with fast MHC isoforms and SERCA2a with MHCI was found in the SOL but not in the PL. An explanation for this disparity may be related to the idea that distinct subsets of MHC-typed fibres are differentially sensitive to neural activation cues mediating the cellular expression of these proteins (Dunn and Michel 1999). On the other hand, Zador et al. (2003 and 2005) showed that over-expression of CAIN, another calcineurin inhibitor, or partial tenotomy prevented the expression of MHCI in regenerating SOL while SERCA2 expression remained elevated. These findings reveal that the regulation of SERCA2 expression is distinct from that of the slow myosin, and that it may be modulated by neuronal activity but is not entirely dependent on it.

The results of the present study also display similar asynchronous changes in the proportion of type I fibres and SERCA2 content. Running alone did not increase the proportion of type I fibres but SERCA2 was significantly increased by 50% (Figure 4-8); however, this did correspond to a substantial increase in type IIA fibres. Cr feeding and running further elevated slow SERCA2 content while the fast-to-slow fibre type transition was attenuated. Interestingly, while Cr feeding alone did not alter the pattern of fibre types, SERCA2 content was remarkably elevated. Thus our data, also clearly show that SERCA2 expression is not tightly linked to slow fibre types.

Similarly, several investigations have suggested that expression of the cytosolic calcium buffering protein, parvalbumin, is under neural control. Denervation (Gundersen et al. 1988) reduces parvalbumin in rat fast-twitch muscles, while cross-reinnervation (Muntener et al. 1987) of the fast-twitch EDL and slow SOL results in respective decreases and increases in parvalbumin. Application of a slow motoneuron-like, low frequency impulse pattern (i.e., CLFS) to rabbit fast-twitch muscle induced a rapid decrease in parvalbumin content (Klug et al. 1983; Leberer and Pette 1986). The decline in parvalbumin content in response to changes in contractile (Pette and Vrbová 1999) activity may be interpreted as a result of the fast-to-slow fibre type conversions (Pette and Vrbová 1992), suggesting that parvalbumin content is under the control of fibre-type-specific programs. In contrast, our data clearly show that Cr loading alone severely reduced (Figure 4-6) parvalbumin protein expression in fast-twitch muscle, and was not further reduced with running as seen in the control run group. Thus our findings indicate that parvalbumin protein expression is not purely dependent on neural regulation. In fact, it may only be indirectly influenced by neural regulation through changes in the intracellular energy potential that typically precede fast-to-slow fibre type transitions (Pette and Staron 1997). Because parvalbumin is largely restricted to type 2D/X and 2B fibres, the dramatic reduction in our study suggests that elevating the capacity for high energy shuttling alleviates the need for Ca^{2+} buffering by parvalbumin. Thus, the intriguing possibility exists that the elevation of slow SERCA2 in response to Cr loading compensates by increasing the efficiency of Ca^{2+} re-uptake. It also follows that enhanced Ca^{2+} re-uptake would sufficiently maintain a lower concentration of free intracellular Ca^{2+} (Pulido et al. 1998), consequently reducing the activation of calcineurin, a Ca^{2+}-dependent protein phosphatase, which stimulates slow fibre-specific gene promoters (Carroll et al. 1999; Naya et al. 2000).

4.4.3 *Metabolic profile*

The aerobic and glycolytic reference enzyme activities measured in this investigation are equilibrium enzymes that exhibit zero kinetics in vivo, and therefore their maximum activity is directly proportional to their enzyme content. The running induced increases in maximal mitochondrial reference enzyme activities, CS and HADH, observed in the present study were similar in magnitude to previous reports of rodent run training (Brannon et al. 1997; Green et al. 1983). Reciprocal reductions in reference enzymes reflecting glycolytic or glycogenolytic capacities also remained virtually unchanged after 13 wk of run training (Brannon et al. 1997; Green et al. 1983). Our novel observation that Cr loading prevented a running induced increase in mitochondrial content in our study is intriguing. The increases in CS and HADH in response to run training alone are also similar to the recent findings that the chronic activation of 5'-AMP-activated protein kinase (AMPK) by the adenosine analogue 5-aminoimidazole-4-carboxamide-1-ß-D-ribofuranoside (AICAR) induces similar changes, which reflect mitochondrial biogenesis that is highly localized within the fast IID/X and IIB fibres (Bamford et al. 2003; Putman et al. 2003). In contrast, the absence of an increase in CS or HADH within our Cr loaded PL muscles after run training is consistent with inhibition of AMPK secondary to a greater average ATP/AMP ratio during running, as well as the direct inhibition of AMPK due to elevated PCr (Ponticos et al. 1998). Indeed it has been shown that energy deprivation within murine skeletal muscles by β-GPA feeding can induce substantial mitochondrial genesis in the presence of AMPK, but not in the muscles of transgenic mice that express an inactive dominant-negative mutant form of AMPK (Zong et al. 2002).

4.4.4 *Conclusions*

Here we report the novel findings that prolonged Cr loading during run training attenuated fast-to-slower fibre type transitions within fast fibre subpopulations, while abolishing related increases in oxidative metabolism.

Despite maintaining a fast glycolytic phenotype, Cr loading alone and with run training severely reduced parvalbumin protein content, elevated slow SERCA2 while preserving fast SERCA1 protein expression. Collectively, our data indicate that Cr loading alters the pattern of protein expression involved in Ca^{2+} regulation, in a manner that is known to lower intracellular Ca^{2+}concentration, and thus appears to preserve the fast glycolytic phenotype.

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CHAPTER 5: Creatine loading attenuates chronic low frequency stimulation induced fibre type transitions in rat skeletal muscle

5.1 INTRODUCTION

Muscle phenotypic properties are influenced by various factors and conditions, which include changes in neuromuscular activity (i.e. electrical stimulation, mechanical loading, exercise training), changes in innervation (denervation/reinnervation) or altered hormonal profile (thyroid hormone). CLFS is a model of muscle training that in rat fast-twitch muscles induces sequential fast-to-slow fibre type conversions in the absence of fibre injury (Pette and Staron 1997; Pette and Vrbová 1992). CLFS has been shown to cause orchestrated changes in gene expression, which include alterations in isoform patterns of contractile myofibrillar proteins from MHCIIb → MHCIId/x → MHCIIa (Ausoni et al. 1990, Leeuw and Pette 1993; Staron et al. 1987; Peuker et al. 1999). The transitions of MHC protein isoforms are preceded by changes in mRNA (Jaschinski et al. 1998). However, the time courses for these changes do not always correspond, and mismatches between MHC mRNA and protein patterns arise (Andersen and Schiaffino 1997). CLFS-induced fast-to-slow fibre type transitions in rat are also associated with a shift from anaerobic to aerobic – oxidative metabolic pathways of energy supply (Pette and Vrbová 1992; Hood and Pette 1989; Reichmann et al. 1985).

Numerous studies using CLFS on rodent models (Carroll et al. 1999; Gundersen et al.1988; Huber and Pette 1996; Klug et al. 1983 and 1988; Leberer and Pette 1986, Ohlendieck et al. 1991) have reported decreases in parvalbumin content concomitant with fast-to-slow fibre type transitions and corresponding prolongation of the excitation-contraction cycle (Carroll et al. 1999; Schwaller et al. 1999). Other studies (Hämäläinen and Pette 1997 and 2001; Leberer et al. 1987 and 1989) have also observed CLFS-induced decreases in the fast Ca^{2+}

ATPase isoform content, SERCA1, and increases in the slow isoform content, SERCA2, within mixed fast-twitch rodent muscles. These adaptations result in higher level of cytosolic Ca^{2+} (Sreter et al. 1987) and a reduced rate of intracellular Ca^{2+} decay (Carroll et al. 1999), and are associated with functional changes in prolonged relaxation profile (increase ½ fall-time) during twitch contractions, resulting in reduced firing frequency to attain fusion during tetanic contractions. Alterations in Ca^{2+} regulation seem to be associated with muscle energy status in vivo (Chin et al. 1995; Duke and Steele 1999).

To date, no studies have investigated whether Cr supplementation, which is known to increase total Cr content in rat (Roy et al. 2002; Brannon et al. 1997) and has the potential to augment the efficiency and capacity for high-energy phosphate shuttling (Wallimann et al. 1998), is able to attenuate forced fast-to-slow fibre type conversions in rodent mixed fast-twitch skeletal muscle. CLFS immediately disrupts PCr and ATP levels (Green and Pette 1997), and thus it makes it an ideal experimental model to study the effects of acute Cr loading on fast-twitch muscle structure, function and metabolism.

Previous work (Gallo et al. 2006, see Chapter 3 and 4) showed that chronic Cr feeding attenuated voluntary wheel running-induced fast-to-slow MHC-based fibre type transitions while increasing fatigue resistance. It was also observed that Cr feeding combined with running minimized increases in the aerobic capacity of rat PL muscle, increased the slow Ca^{2+} ATPase isoform content, SERCA2, while dramatically reducing parvalbumin protein expression. All together, these findings show that chronic Cr loading during run training maintains a faster fibre muscle phenotype, prevents increases in aerobic activity, while also enhancing the potential for improved intracellular Ca^{2+} regulation. By comparison CLFS should induce a similar but more pronounced fast-to-slow fibre type conversion compared with voluntary wheel running. The TA muscle was the

primary focus of this investigation because it possesses a mixed fast-twitch MHC-based phenotype and is innervated by the common peroneal nerve which can be easily targeted by CLFS. The purpose of the present study is to test the hypothesis that acute Cr feeding during CLFS will maintain the fast, glycolytic MHC-based phenotype of the fast-twitch TA muscle. It was further hypothesized that Cr feeding during CLFS would result in higher levels of parvalbumin and fast SERCA1, and low levels of slow SERCA2 compared with a stimulated group that received no Cr.

5.2 MATERIALS AND METHODS

5.2.1 *Animals and experimental design*

Twenty-four male Sprague-Dawley rats weighing 236.5 ± 3.51 g (mean ± S.E.M.) were used in this study. Animals were obtained from an inbred colony maintained at the University of Alberta Health Sciences Laboratory Animal Services and all experiments received ethical approval from the Health Sciences Animal Welfare and Policy Committee and were completed in accordance with the guidelines of the Canadian Council for Animal Care. Animals were individually housed under controlled environmental conditions (22°C and 12-h alternating light and dark cycles), and ingested standard rat chow and their respective drinking solutions ad libitum. The animals were acclimatized to the facility, cages, rodent diet and their drinking solutions for 3 days before starting the study. Body mass, food and solution intake were monitored throughout the investigation.

Animals were randomly assigned to one of the following 4 groups: (1) creatine (Cr) fed + CLFS applied to the left leg (Cr-Stim, n = 6), (2) only CLFS applied to the left leg (Con-Stim, n = 6), (3) Cr fed + left leg sham operation (Cr-

Sham, n = 7) or (4) left leg sham operation only (Con-Sham, n = 5). The Cr groups (1 and 3) were fed a 1% (w/v) Cr solution in 5% (w/v) dextrose while the Con groups (2 and 4) consumed a solution of 5% (w/v) dextrose.

5.2.2 *Surgery and chronic low frequency stimulation*

Following 3 days of acclimatization, bipolar electrodes were implanted under general anaesthesia [mixture of ketamine (75 mg/kg body wt), xylazine (8 mg/kg body wt) and acepromazine maleate (0.5 mg/kg body wt)] lateral to the common peroneal nerve of the left hindlimb, externalized at the dorsal inter-scapular region, and connected to a small portable stimulator; seven days were allowed for recovery (see Figure 1-6 for schematic of CLFS model). To ensure muscle Cr loading, the Cr fed groups consumed a solution containing 1% (w/v) Cr and 5% (w/v) dextrose solution for 10 days before CLFS was initiated and throughout the remainder of the study. Following recovery from surgery, CLFS (10Hz, stimulus width 380 *u*s, 10 hr/day) was applied across the left common peroneal nerve as previously described (Simoneau et al. 1988; Putman et al. 2004) for 10 consecutive days. The stimulated left TA muscles were studied and the right TA muscles served as the internal contralateral controls.

5.2.3 *Isometric functional measurements and muscle sampling*

After 10 days of CLFS or sham, animals were anaesthetized as described above. Incisions were made along the dorsum of the right and left hindlimbs and the ankle tendons of the TA muscles were separated and individually tied with a 2.0 silk, while the EDL was denervated. A silastic nerve cuff embedded with 2 multistranded stainless steel wires was positioned around the sciatic nerve for electrical stimulation. The 2.0 silk was then attached to a Kulite strain gauge (model KH-102), for sequential force recordings. Before completing the isometric

functional measures, skin incisions were loosely sutured. Throughout the experiment, animals were in a prone position and secured with clamps at the ankle and knee joints. Core body temperature was continuously monitored with a rectal probe and maintained at 37°C with a heating pad.

Isometric muscle contractile function measures were completed as previously described by Gallo et al. (2004) (see Chapter 2.2.2). Before recording the measures, the optimal resting length (L_o) required to generate maximum twitch force was established for each muscle. Maximum twitch (TW_t: mN) forces, time-to-peak tension (TTP: ms), half-rise time (½RT: ms), and half-fall time (½FT: ms) were measured in the both the left and right TA muscles.

Upon completion of the study, TA muscles were collected under heavy anaesthesia (75 mg/kg body wt ketamine and 8 mg/kg body wt xylazine) from both hindlimbs, weighed, fixed in a slightly stretched position, frozen in melting isopentane (-159°C) cooled in liquid nitrogen (N_2, -196°C). Animals were euthanized with an overdose of Euthanyl (100mg·kg body wt^{-1}) followed by exsanguination. Muscles were stored in liquid N_2 until analysis.

5.2.4 *Total creatine levels*

Portions of each TA muscle were lyophilized and the extracts were analyzed as previously described in Chapter 3.2.3. The neutralized supernatant was used for biochemical determination of intramuscular TCr concentration.

5.2.5 *Electrophoresis and real-time reverse transcriptase polymerase chain reaction of myosin heavy chain isoform content*

The relative MHC isoform contents within TA muscles were analyzed as previously described in Chapter 2.2.3. Patterns of MHC isoform expression were further analyzed at the mRNA level using real-time RT-PCR. RNA extraction was performed according to an established procedure (Vinsky et al. 2007). The concentrations and purity of the RNA extracts were evaluated by measuring the absorbance at 260 and 280 nm, respectively, using a NanoDrop ND 1000 system. The cDNA synthesis was performed according to an established procedure (Bamford et al. 2003). Briefly, random oligo (dT_{15}) primers and Moloney murine leukemia virus (M-MLV) DNA polymerase were added to diluted samples (1 µg/µl) and reverse transcription was performed for 1 hr at 37°C. Primers and Taqman-MGB probes were designed with EMBL-EBI and aligned using Clustal W for rat MHCIβ (X15939), MHCIIa (L13606), MHCIId/(x) (XM 213345) and MHCIIb (L24897) (Table 1). Real-time PCR was performed on 1 µl cDNA sample, in duplicate, using an ABI 7900HT thermocycler. Real-time semi-quantification of MHC isoforms for each sample was performed and the mean C_t value was normalized against its respective 18S (endogenous control) C_t value using the standard ΔC_t method (Pfaffl et al. 2001). Inter-assay variation was evaluated by repeated analysis of a known sample on each 96-well plate and confirmed to be negligible.

5.2.6 *Western blot analyses of parvalbumin and calcium ATPases*

Western blot analyses of parvalbumin and Ca^{2+} ATPases in the TA muscles were performed as described in Chapter 4.2.3. Membranes probed for SERCA2 were reprobed with monoclonal anti-desmin (clone DE-U-10) (1:500 in blocking solution), which served as the internal control. Immunoreactivity for

desmin (50-55 kDa) was visualized with the Amersham Biosciences Detection Reagents after incubation with anti-mouse IgG (1:2,000 in blocking solution), and corresponded to a molecular weight of 50-55 kDa, as determined by comparison against standard molecular weight markers (Precision Plus Protein Standards).

5.2.7 *Metabolic enzyme analyses*

For measurement of citrate synthase (CS, EC 4.1.3.7), 3-hydroxyacyl-CoA dehydrogenase (HADH, EC 1.1.1.35) and glyceraldehyde phosphate dehydrogenase (GAPDH, EC 1.2.1.12) activities in TA muscles refer to Chapter 4.2.4. For measurements of creatine kinase activity (CK, EC 2.7.3.2), portions of TA muscles were extracted in 50mM glycylglycine, 5mM DTT, 1mM EDTA and 0.1% Triton X-100 (pH 7.4) (Sigma Technical Bulletin). CK activities were subsequently measured at 30°C according to Lowry et al. (1978).

5.2.8 *Statistical analyses*

Data are summarized as mean ± SEM. Differences between group means were assessed using a Two-Way Analysis of Variance (treatment-treatment interaction: creatine and CLFS). When a significant F-ratio was found, differences were located using the LSD post-hoc analysis for planned comparisons. Differences between left and right legs within groups were assessed using a paired sample Student's T-test. Differences were considered significant at $P < 0.05$, but actual P-values are cited.

5.3 RESULTS

5.3.1 *Body, muscle and muscle-to-body mass ratio*

Figure 5-1 illustrates gains in body mass were similar for all treatment groups and did not differ from one another. All treatment groups gained approximately 117 ± 5.1g throughout the study. The mean body masses in the Cre-Sham, Cre-Stim, Con-Sham and Con-Stim groups were 359 ± 8.1g, 331 ± 9.2g, 366 ± 11.3 and 351 ± 13.5 g, respectively. The masses of TA muscles (Table 5-2) were not different within or between groups. The TA-to-body mass ratio of the Cre-Stim group was, however, greater ($P < 0.02$) than that of Con-Stim (Table 5-2). The wet-to-dry ratio was also measured to evaluate muscle hydration status. The wet-to-dry ratio of TA muscles was not affected by CLFS or Cr loading (Table 5-3). However, in the stimulated muscles, the wet-to-dry ratio of the Con-Stim was higher than that of Cre-Stim and Con-Sham ($P < 0.02$).

5.3.2 *Solution intake, creatine consumption and total creatine levels*

Total solution intake (Figure 5-2) in the Cre-Stim, Cre-Sham, Con-Stim and Con-Sham groups were 934±124.6mL, 1005±117.3mL, 1517±510.6mL and 1377±349.7mL, respectively. Total solution intake of Cr groups was 33% lower than Con groups (967±121.8mL vs. 1453±429.7mL, $P < 0.001$). Figure 5-3 shows that daily Cr ingestion did not differ between Cre-Stim and Cre-Sham groups during 20 days of Cr loading. Daily averaged Cr consumption was 1.84±0.074g/kg in Cre-Stim and 2.15±0.181g/kg in Cre-Sham. Figure 5-4 illustrates TCr content of Cr and Con groups. TCr was elevated by 21% ($P < 0.03$) in the Cr fed groups compared with the dextrose fed (Con) groups (Cr fed vs. dextrose fed: 160.4±45.03 vs. 126.1±51.5momI/kg dry wt).

5.3.3 *Isolated isometric measures of force and contraction speed*

Measures of muscle strength (TW_f) and contractile speed properties (TTP, ½ RT, ½ FT) of the TA are shown in Figure 5-5. Neither of the Cr or CLFS treatments alone had any significant effects on TW_f (Figure 5-5A). In the Cre-Stim group, however, the stimulated TA produced 16% less TW_f compared to contralateral control muscles (2857±218.4mN vs. 3322±181.2mN) ($P < 0.008$). Cr feeding alone increased the TTP (Figure 5-5B). This was reflected by an increase of 6.1ms in the Cre-Sham compared to Con-Sham ($P < 0.05$) and by 9.6ms in Cre-Stim compared to Con-Stim group ($P < 0.02$). Cr feeding alone led to a 2.7ms increase ($P < 0.04$) in the ½ RT in the Cre-Sham compared to Con-Sham group (Figure 5- 5C). When Cr loading was combined with stimulation the ½ RT was also elevated (Cre-Stim vs. Con-Stim, $P < 0.05$). The ½ FT was not altered by Cr feeding alone (Figure 5- 5D). However, CLFS increased the ½ FT by 13.7ms in the Con-Stim compared to Con-Sham group ($P < 0.05$).

5.3.4 *Myosin heavy chain isoform content and gene expression*

The method used to isolate and quantify MHC isoforms and the corresponding data are shown in Figures 5-6 and 5-7, respectively. The TA displayed substantial fast-to-slow MHC-isoform transitions in response to CLFS. CLFS alone (Con-Stim) increased MHCI and MHCIIa by 2% (Figure 5-7A, $P < 0.006$) and 15.2%, respectively (Figure 5-7B, $P < 0.0005$), and decreased MHCIIb by 22% (Figure 5- 7D, $P < 0.004$) compared to its contralateral TA muscles and compared to Con-Sham group. When Cr loading was combined with CLFS, the increases in MHCI and MHCIIa and decreases in MHCIIb were attenuated ($P < 0.03$). CLFS combined with Cr loading increased MHCIId/x by 6% (Figure 5-7C, $P < 0.03$).

Figure 5-8 shows the mRNA content of the TA as quantified by real-time RT-PCR. The ΔCt values reported are inversely related to the level of gene expression (Pfaffl et al. 2001). No significant changes were observed in MHCI mRNA content between any of the treatment groups. CLFS treatment greatly increased MHCIIa and decreased MHCIIb mRNA content in the Cre and Con groups, as reflected by lower and higher ΔCt values, respectively (Figure 5-8C and 5-8D) ($P < 0.03$). Cr combined with CLFS resulted in higher MHCIIb mRNA content compared to CLFS alone, as reflected by a lower ΔCt value (ie. 6.1 vs. 9.1; Figure 5-8D, $P < 0.008$). Similarly, application of CLFS and Cr loaded muscles maintained higher MHCIId/x mRNA content compared with CLFS alone, as reflected by a lower ΔCt value (i.e.6.2 vs. 8.5; Figure 5-8C, $P < 0.04$).

5.3.5 *Parvalbumin and calcium ATPase content*

Figure 5-9A illustrates the method used to quantify parvalbumin content. Neither CLFS nor Cr treatments had any effect on parvalbumin content Parvalbumin content in left legs (stimulated or sham) was not different in all groups compared to C ($P \geq 0.26$) (Figure 5-9B).

The semiquantitative methods used to compare the relative abundance of SERCA1 and SERCA2 isoforms of the Ca2+ ATPase in TA muscles are illustrated in Figures 5-10 and 5-11, respectively. CLFS decreased the content of the fast Ca^{2+} ATPase isoform, SERCA1, in the Con-Stim group compared to C (Figure 5-10B) ($P < 0.03$). SERCA1 content did not statistically change in response to CLFS in the Cre-Stim group ($P = 0.80$). No differences in SERCA1 content were observed between groups. The slow Ca^{2+} ATPase, SERCA2, content was not affected by any of the treatments (Figure 5-11B).

5.3.6 *Oxidative and glycolytic enzyme content*

All enzyme activities are shown in Figure 5-12. Enzyme activities in the TA are within the ranges reported by Bamford et al. (2003) and Lowry et al. (1980). In the present study, CLFS increased the CS and HADH activities by 1.7 and 1.6-fold in the Cre group ($P < 0.02$) and 1.9 and 1.4-fold in the Con group, respectively (Figure 5-12A and B, $P < 0.04$). It is noteworthy to report that GAPDH activity was 29% higher in the Cre-Stim compared to Con-Stim ($P < 0.05$) and that CLFS alone decreased CK activity by 1.3 and 1.2-fold in the Cre and Con groups, respectively (Figure 5-12E, $P< 0.05$).

Table 5-1

Rat specific real-time polymerase chain reaction primers and probes

Target	Forward (5'-3')	Reverse (5'-3')	Probe (5'-3')
MHCI	GCAGTTGGATGAGCGACTCA	TCCTCAATCCTGGCGTTGA	AGAAGGACTTTGAGTTAAAT
MHCIIa	GGCGGCAAGAAGCAGATC	TTCCGCTTCTGCTCACTCTCT	AGGCCAGAGTGCGTG
MHCIId/x	GGCGGCAAGAAGCAGATC	TTCGTTTTCAACTTCTCCTTCAAGT	AGGCCAGGGTCCG
MHCIIb	GGCGGCAAGAAGCAGATC	TTTTCCACCTCGTTTTCAAGCT	TGGAGGCCAGAGTGA

Table 5-2

Muscle mass (mg) and tibialis anterior-to-body mass ratio in control and stimulated/sham hindlimbs after creatine feeding and chronic low frequency stimulation treatment

Group	Control TA	Stim/Sham TA	Control TA-to-body mass ratio	Stim/Sham TA-to-body mass ratio
Cre-Stim	639 ± 14.5	616 ± 13.1	1.93 ± 0.04 [a]	1.87 ± 0.05
Cre-Sham	661 ± 19.2	665 ± 16.9	1.85 ± 0.05	1.86 ± 0.07
Con-Stim	622 ± 35.8	627± 26.9	1.79 ± 0.04	1.79 ± 0.03
Con-Sham	641 ± 21.2	670 ± 32.9	1.71 ± 0.05	1.80 ± 0.11

Data are mean ± S.E.M. "a" indicates Cre-Stim is different from Con-Stim.

Table 5-3

Wet-to-dry ratio of tibialis anterior in control and stimulated/sham hindlimbs after creatine feeding and chronic low frequency stimulation treatment

Group	Control TA	Stim/Sham TA
Cre-Stim	5.26 ± 0.27	5.11 ± 0.33 [a]
Cre-Sham	5.27 ± 0.36	5.19 ± 0.31
Con-Stim	5.97 ± 0.49	6.16 ± 0.59 [d]
Con-Sham	5.12 ± 0.23	4.78 ± 0.27

Data are mean ± S.E.M. "a" indicates Cre-Stim is different from Con-Stim. "d" indicates Con-Stim is different from Con-Sham.

Figure 5-1

Changes in body mass during 20 days of creatine loading. CLFS was applied during the last 10 days of Cr loading. "*" indicates Cre-Sham is different from all other groups. Differences between group means were assessed using a Two-Way ANOVA for each time point.

Figure 5-1

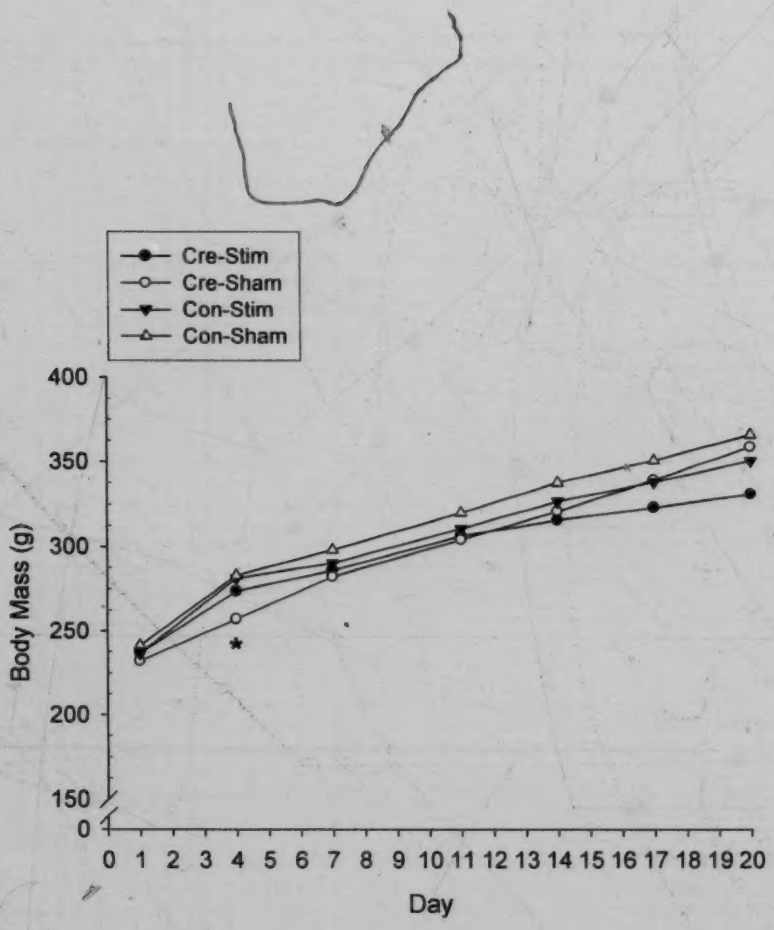

Cre-Stim
Cre-Sham
Con-Stim
Con-Sham
Body Mass (g)
400
350
300
250
200
150
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Day
*

Figure 5-2

Daily solution intake during 20 days of creatine loading. "a" indicates Cre-Stim is different from Con-Stim. "b" indicates Cre-Sham is different from Con-Sham. "d" indicates Con-Stim is different from Con-Sham. Differences between group means were assessed using a Two-Way ANOVA for each time point.

Figure 5-2

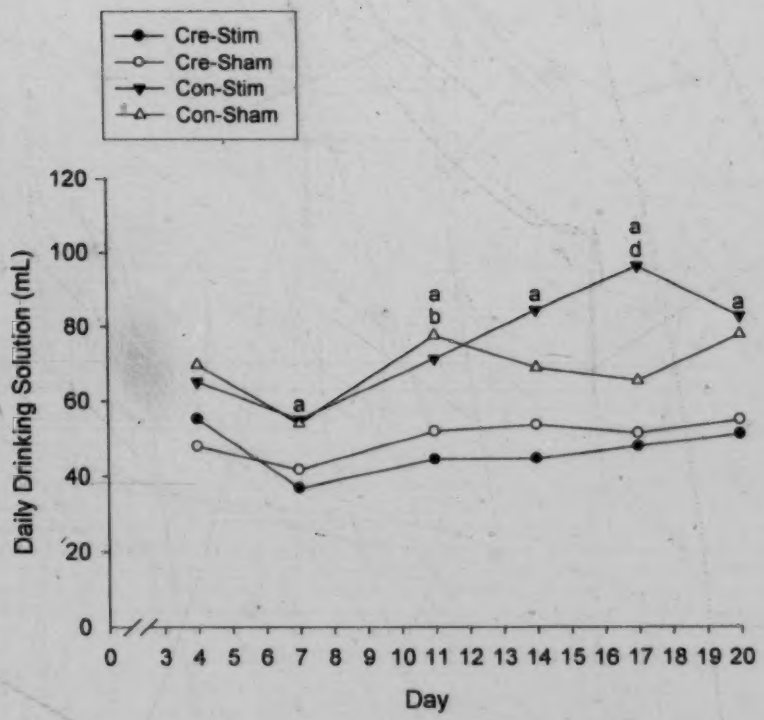

Cre-Stim
Cre-Sham
Con-Stim
Con-Sham
Daily Drinking Solution (mL)
120
100
80
60
40
20
0
0 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Day
a
a
b
a
a
d
a

Figure 5-3

Daily creatine consumption in the Cre-Stim and Cre-Sham groups during 20 days of creatine loading. Differences between group means were assessed using a One-Way ANOVA for each time point.

Figure 5-3

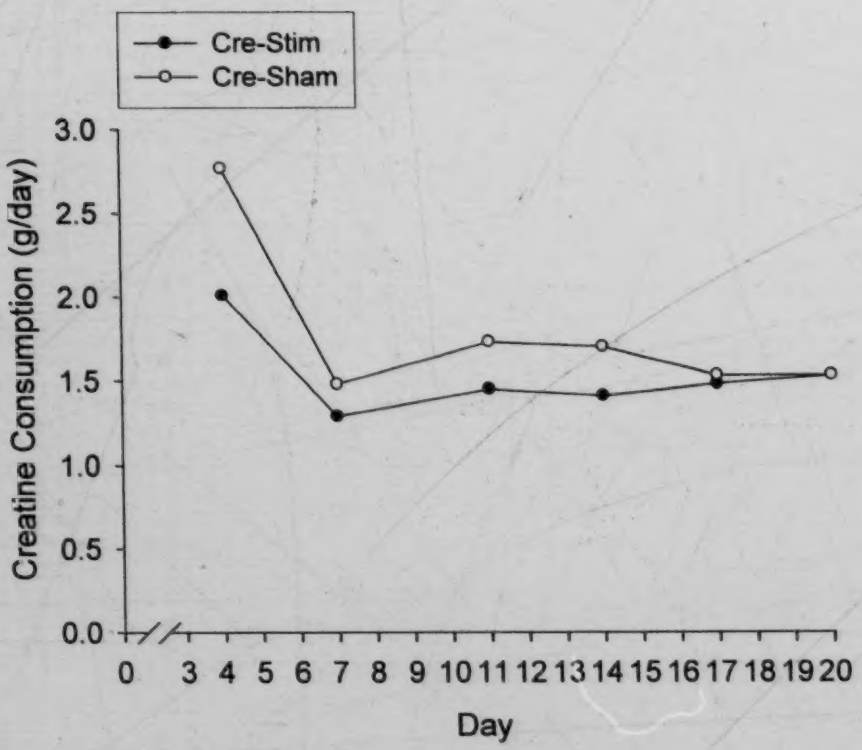

Cre-Stim
Cre-Sham
Creatine Consumption (g/day)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Day

Figure 5-4

Total creatine content within the tibialis anterior muscles of creatine and dextrose (Control) fed groups. "*" indicates Cr fed groups are different from dextrose fed groups. Differences between group means were assessed using a One-Way ANOVA. TCr levels of Cr-Sham and Cr-Stim were not statistically different (as assessed by a One-Way ANOVA), and therefore data were pooled. Similarly, TCr levels of Con-Sham and Con-Stim groups were pooled.

Figure 5-4

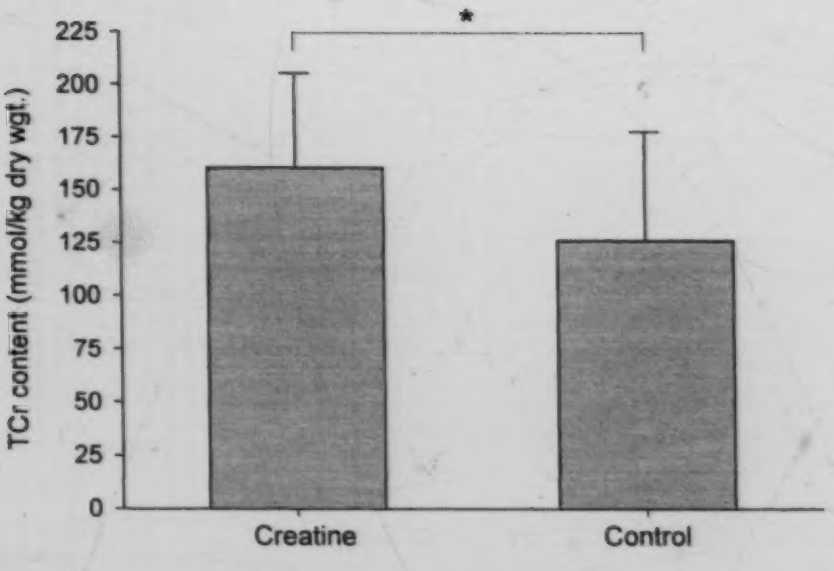

*
225
200
175
150
125
100
75
50
25
0
TCr content (mmol/kg dry wgt.)
Creatine
Control

Figure 5-5

Isometric functional measures of the tibialis anterior muscles. A) Isometric twitch force (TW_f), (B) time-to-peak tension, (C) half-rise time, and (D) half-relaxation time. "a" indicates Cre-Stim is different from Con-Stim. "b" indicates Cre-Sham is different from Con-Sham. " d" indicates Con-Stim is different from Con-Sham. "*" indicates contralateral control TA is different from stimulated/sham TA. Differences between group means were assessed using a Two-Way ANOVA.

Figure 5-5

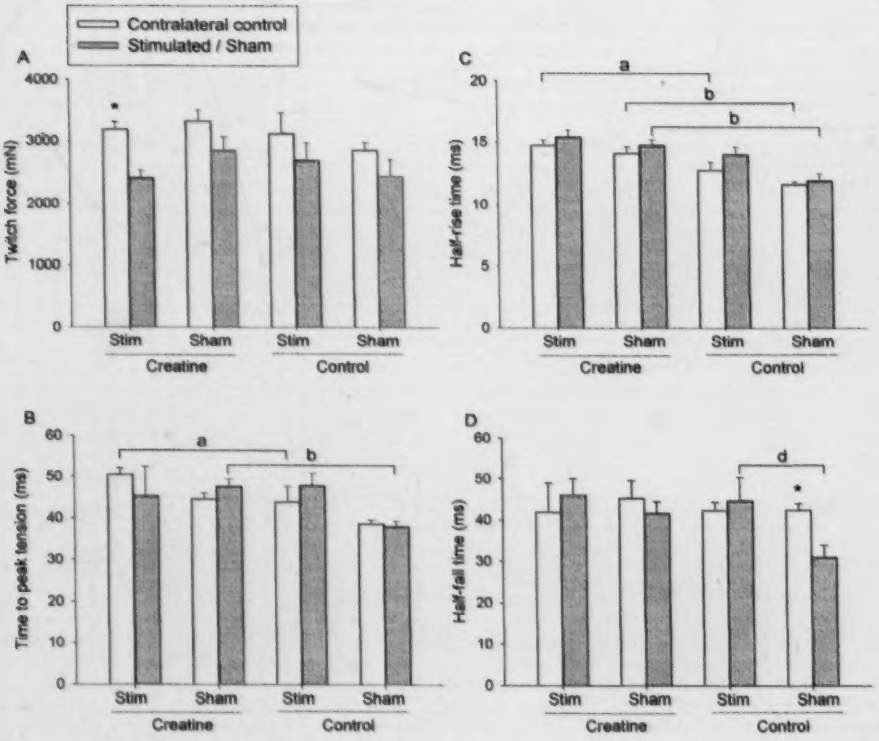

Contralateral control
Stimulated / Sham
A
Twitch force (mN)
4000
3000
2000
1000
0
*
Stim
Sham
Stim
Sham
Creatine
Control
C
Half-rise time (ms)
20
15
10
5
0
a
b
b
Stim
Sham
Stim
Sham
Creatine
Control
B
Time to peak tension (ms)
60
50
40
30
20
10
0
a
b
Stim
Sham
Stim
Sham
Creatine
Control
D
Half-fall time (ms)
60
50
40
30
20
10
0
d
*
Stim
Sham
Stim
Sham
Creatine
Control

Figure 5-6

Electrophoretic method used to separate and quantify myosin heavy chain isoform composition of tibialis anterior muscles. Control and Stimulated are shown.

Figure 5-6

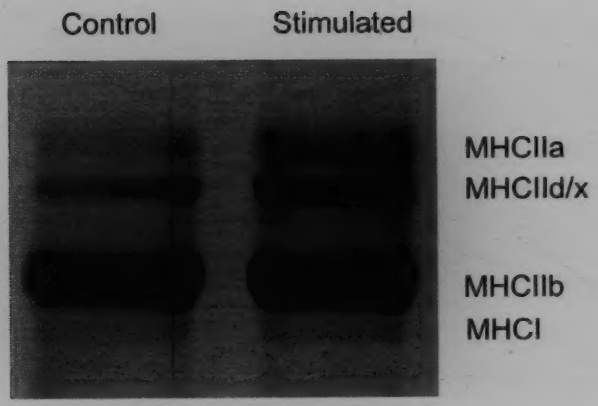


Figure 5-7

Myosin heavy chain isoform composition of tibialis anterior muscles: MHCI content (A), MHCIIa (B), MHCIId/x (C) and MHCIIb (D). "a" indicates Cre-Stim is different from Con-Stim. "b" indicates Cre-Sham is different from Con-Sham. "c" indicates Cre-Stim is different from Cre-Sham. "d" indicates Con-Stim is different from Con-Sham. "*" indicates contralateral control TA is different from stimulated/sham TA. Differences between group means were assessed using a Two-Way ANOVA.

Figure 5-7

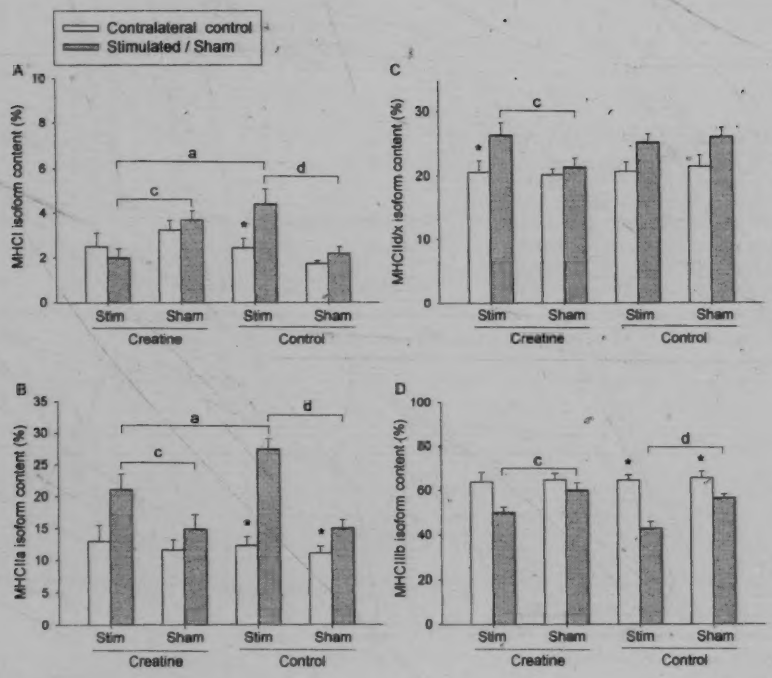

Contralateral control
Stimulated / Sham
A
MHCI isoform content (%)
B
MHCIIa isoform content (%)
C
MHCIIdx isoform content (%)
D
MHCIIb isoform content (%)
Stim
Sham
Creatine
Control
a
c
d
*

Figure 5-8

Myosin heavy chain mRNA content (ΔCt) assessed via real-time polymerase chain reaction in the tibialis anterior muscles: MHCI mRNA (A), MHCIIa mRNA (B), MHCIId/x mRNA (C) and MHCIIb mRNA (D). "a" indicates Cre-Stim is different from Con-Stim. "d" indicates Con-Stim is different from Con-Sham. ΔCt values are inversely related to the level of gene expression. "*" indicates contralateral control TA is different from stimulated/sham TA. Differences between group means were assessed using a Two-Way ANOVA.

Figure 5-8

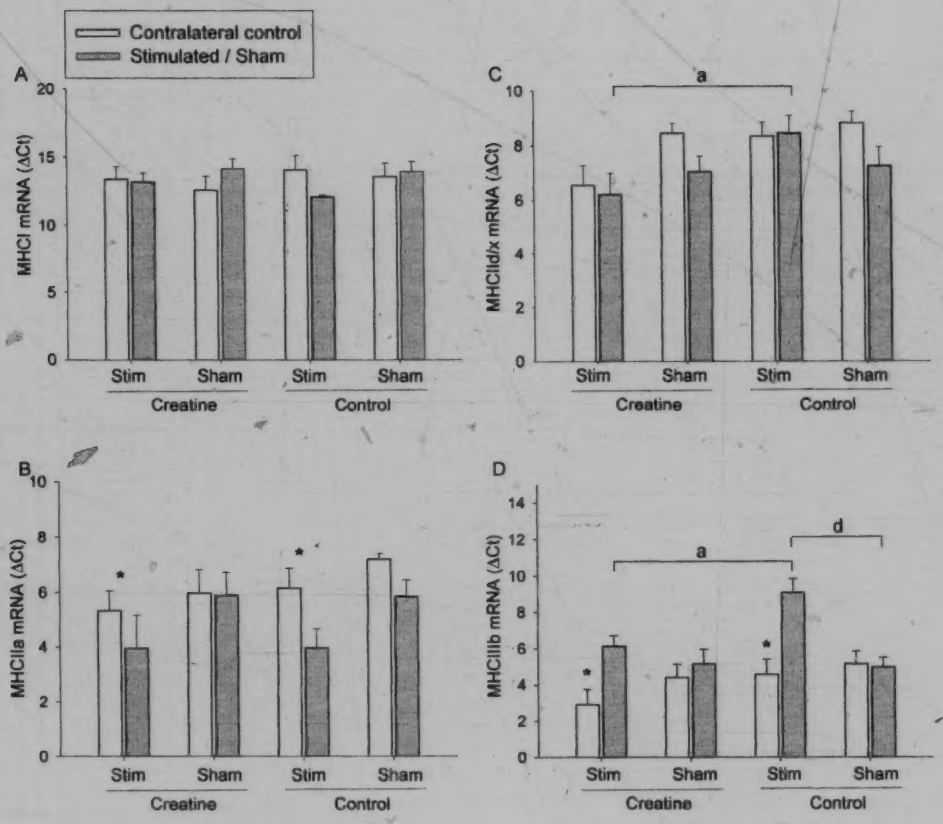
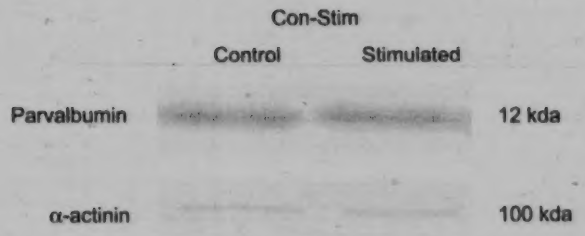

Contralateral control
Stimulated / Sham
A
MHCI mRNA (ΔCt)
20
15
10
5
0
Stim
Sham
Stim
Sham
Creatine
Control
C
MHCIId/x mRNA (ΔCt)
10
8
6
4
2
0
a
Stim
Sham
Stim
Sham
Creatine
Control
B
MHCIIa mRNA (ΔCt)
10
8
6
4
2
0
*
*
Stim
Sham
Stim
Sham
Creatine
Control
D
MHCIIIb mRNA (ΔCt)
14
12
10
8
6
4
2
0
a
d
*
*
Stim
Sham
Stim
Sham
Creatine
Control

Figure 5-9

A representative immunoblot (A) and densitometric evaluation (B) of pavalbumin in the tibialis anterior muscles. Units of measure: parvalbumin / α-actinin (loading control). "c" indicates Cre-Stim is different from Cre-Sham. Differences between group means were assessed using a Two-Way ANOVA.

Figure 5-9

A


Con-Stim
Control
Stimulated
Parvalbumin
12 kda
α-actinin
100 kda

B

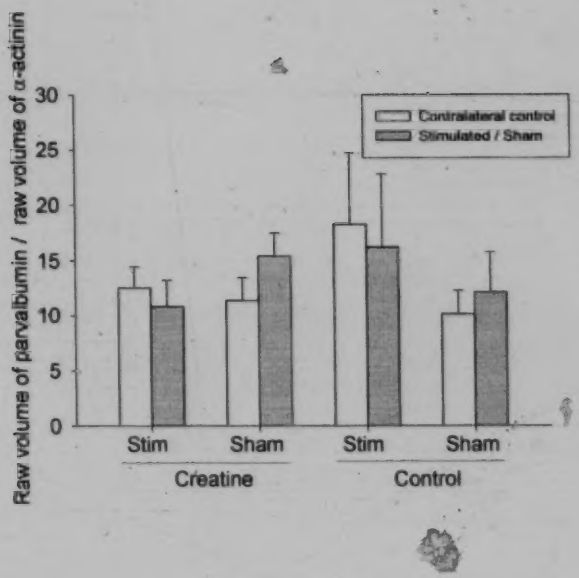
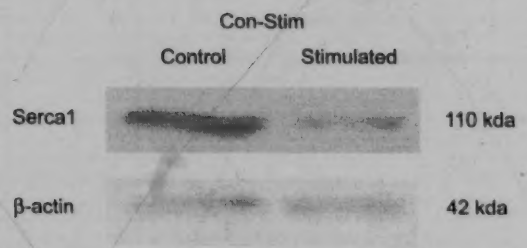

Raw volume of parvalbumin / raw volume of α-actinin
30
25
20
15
10
5
0
Contralateral control
Stimulated / Sham
Stim
Sham
Stim
Sham
Creatine
Control

Figure 5-10

A representative immunoblot (A) and densitometric evaluation (B) of SERCA1, in the tibialis anterior muscles. Units of measure: SERCA1 / β-actin (loading control). "*" indicates contralateral control TA is different from stimulated/sham TA. Differences between group means were assessed using a Two-Way ANOVA.

Figure 5-10

A



B

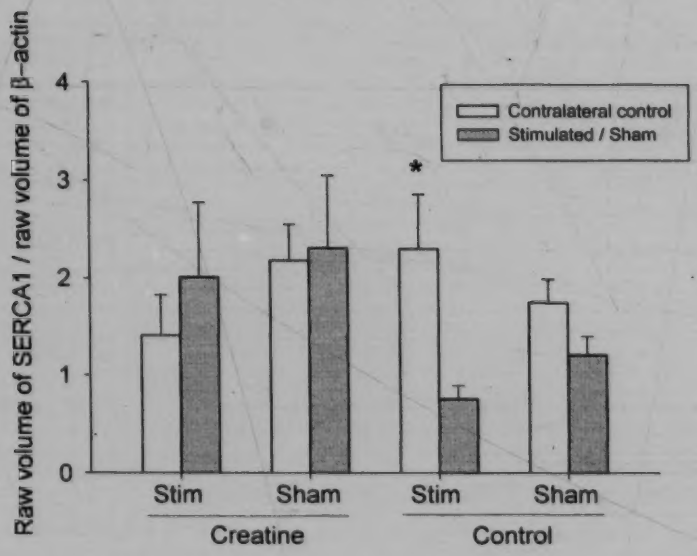
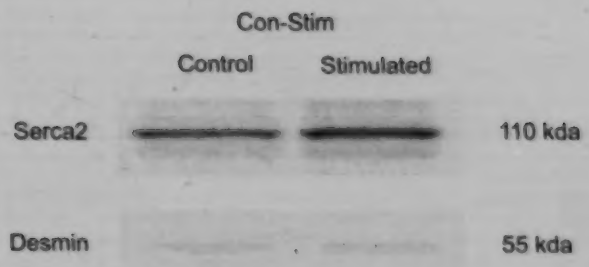


Figure 5-11

A representative immunoblot (A) and densitometric evaluation (B) of SERCA2, in the tibialis anterior muscles. Units of measure: SERCA2 / desmin (loading control). Differences between group means were assessed using a Two-Way ANOVA.

Figure 5-11

A



B

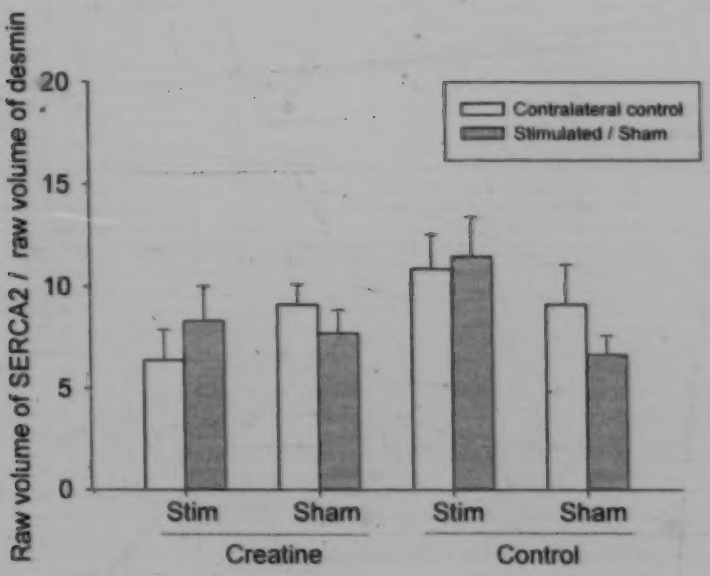
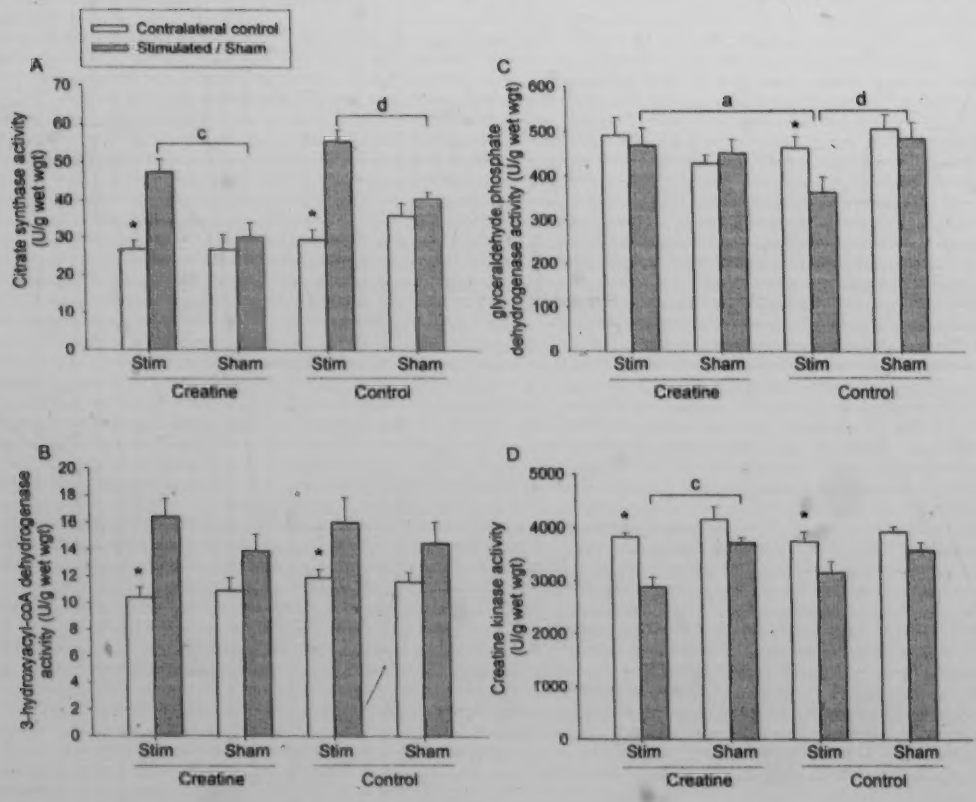


Figure 5-12

Maximum activities of citrate synthase, 3-hydroxyacyl –CoA dehydrogenase, glyceraldehydephosphate dehydrogenase and creatine kinase in the tibialis anterior muscles. Units of measure are U/g wet weight. "a" indicates Cre-Stim is different from Con-Stim. "c" indicates Cre-Stim is different from Cre-Sham. "d" indicates Con-Stim is different from Con-Sham. "*" indicates contralateral control TA is different from stimulated/sham TA. Differences between group means were assessed using a Two-Way ANOVA.

Figure 5-12


Contralateral control
Stimulated / Sham
A
Citrate synthase activity (U/g wet wgt)
c
d
*
Stim
Sham
Stim
Sham
Creatine
Control
C
glyceraldehyde phosphate dehydrogenase activity (U/g wet wgt)
a
d
*
Stim
Sham
Stim
Sham
Creatine
Control
B
3-hydroxyacyl-coA dehydrogenase activity (U/g wet wgt)
*
Stim
Sham
Stim
Sham
Creatine
Control
D
Creatine kinase activity (U/g wet wgt)
c
*
Stim
Sham
Stim
Sham
Creatine
Control

5.4 DISCUSSION

CLFS is an ideal model of muscle endurance training used to study the adaptive potential of skeletal muscle fibre, and in particular the relationships between the functional, metabolic and molecular characteristics (Pette and Vrbová, 1992). This study investigated whether acute Cr loading would attenuate CLFS-induced MHC-isoform transitions, alter the corresponding isometric contractile properties, the proteins that regulate intracellular Ca^{2+} levels and the metabolic profile in the rat TA muscle. The novel findings of this experiment report that acute Cr loading during CLFS is able to maintain a faster MHC-based phenotype as determined at the protein and mRNA levels, and an enhanced glycolytic capacity as reflected by higher activity of the reference enzyme GAPDH. However, Cr loading alone did not dramatically alter isometric functional properties or the contents of the Ca^{2+} regulatory proteins.

5.4.1 MHC-based fibre type transitions and functional measures

CLFS has been shown to induce the transformation of fast-twitch fibres into slower contracting and more oxidative phenotypes in rodent skeletal muscles (Ausoni et al. 1990; Leeuw and Pette 1993; Staron et al. 1987; Peuker et al. 1999). Delp and Pette (1994) found that 28 days of CLFS in the rat EDL decreased the percentage of type-IIB fibres by 43%, and increased the proportions of type-IIA (23%) and type-I (4%) fibres with stimulation up to 60 days. Similarly, Termin et al. (1989) showed that in rat fast-twitch skeletal muscles, CLFS caused partial fibre type transformation as displayed by an increase in MHCIIa with corresponding decreases in MHCIId/x and MHCIIb isoforms, without induction of the slowest MHCI isoform. The MHC-isoform based fibre type transitions in the current study as quantified by gel electrophoresis reveal that CLFS led to increases in MHCI (2%) and MHCIIa (15%) and decreases in MHCIIb (22%) content. These results agree with numerous studies where it is generally shown that mammalian skeletal muscle

changes its MHC-based fibre phenotype in response to CLFS. Additionally, the application of CLFS led to MHC-based alterations at the mRNA level. CLFS increased MHCIIa and decreased MHCIIb mRNA content as quantified by real-time PCR. Jaschinski et al. (1998) also found increases in MHCIIa mRNA and decreases in MHCIIb mRNA after 3 days and 1 day of CLFS, in rat EDL, respectively. The changes at the protein level, however, remained restricted to the MHCIIb to MHCIIa transition, thus agreeing that the induced alterations in MHC isoform expression resulted from changes in pre-translational activities.

Cr feeding combined with CLFS was able to maintain higher proportions of MHCIId/x and MHCIIb. The cellular signals initiating these transformation processes are not fully identified. Nonetheless, it is reasonable to believe that altered concentrations of key metabolites of energy supply (i.e. TCr levels), may play an important role in prompting the transformation progression. Henriksson et al. (1988) found that ATP, glycogen and PCr levels were reduced with long-term stimulation in rabbit TA. Similarly, Green et al. (1992) showed that 10 days of CLFS (24hrs/day, 10Hz) remarkably decreased levels of ATP, TCr and PCr levels in rabbit TA muscle, and concluded that a decreased phosphorylation potential of the adenylate system may be a crucial signal eliciting muscle fibre type transformations. Conjard and Pette (1999) showed that changes in energy-rich phosphate levels differ between fibre type and display fibre specific responses to 8 days of CLFS in rabbit TA muscle. They concluded that PCr can be used as a sensitive biochemical marker of the degree of contractile activity in single fibre muscles. The 8-day values of the ATP phosphorylation potential were reduced and according to their hypothesis, this reduction may act as an important signal initiating fibre type transition (Conjard et al. 1998; Green et al. 1992).

Cr feeding attenuated the CLFS-induced increases in the slow MHC isoforms and decreases in the fast MHC isoforms at the protein and mRNA level as reflected by the maintenance of higher MHCIId/x and MHCIIb protein contents

and MHCIId/x and MHCIIb mRNA contents, respectively. The results from the present study, agree with the MHC-based isoform transitions seen with Cr feeding and 13 weeks of voluntary running where lower MHCIIa and MHCIId/x contents and greater MHCIIb content in the Cr group compared to the control group (see Chapter 3). In accordance with these findings Cr feeding combined with voluntary running led to a 10% decrease in type IIA and an 11% increase in type IIB fibres compared with control (see Chapter 4). All together, two distinct endurance exercise regimens resulted in similar fast-to-slow MHC based fibre type transitions in mixed fast-twitch rat muscle. Cr feeding combined with either long-term (13 weeks) wheel running or short-term (10 days) CLFS attenuated the activity-induced fast-to-slow MHC based fibre transitions in PL and TA muscles, respectively.

Cr feeding alone surprisingly increased TTP and ½ RT by 9.6ms and 2.7ms, respectively in Cre-Sham compared to Con-Sham. The ½ FT was not altered by Cr feeding alone; however, CLFS increased the ½ FT by 13.7ms in the Con-Stim compared to Con-Sham group. Therefore, there seems to be a disconnect between changes in muscle fibre type and the corresponding contractile properties. This appears to be related to the adaptive changes within the proteins that regulate intracellular calcium as will be discussed below. Some factors that influence functional measures upstream of excitation-contraction coupling could account for the slower speed measures of contraction and relaxation despite the attenuation of activity-induced fast-to-slow MHC-based fibre type transitions. Leeuw and Pette (1993 and 1996) have reported that troponin T isoforms and myosin light chain (MLC) isoforms could exist in atypical combinations and affect specific functional requirements. For example, due to an earlier onset of the fast-to-slow transition of the regulatory light chains and the delayed fast-to-slow exchange of the alkali light chains, a spectrum of hybrid combinations result, and may explain the differences in functional measures. A reduction in the MLC_{3f} to MLC_{2f} ratio could also lead to decreased speeds of

contraction (Bottinelli et al. 1994), and thus counteract the functional consequences of Cr's attenuation of fast-to-slow fibre type transitions.

5.4.2 *Parvalbumin content*

Numerous studies have observed reductions in the expression of the cytosolic Ca^{2+} buffering protein, parvalbumin, in response to CLFS (Klug et al. 1983; Huber and Pette 1996; Leberer and Pette 1986). Most CLFS studies to date have used a rabbit model of 24hrs stimulation at 10Hz. In contrast, we used a rat model of 10hrs daily stimulation at 10Hz, which is known to induce transformation at a significantly slower rate compared to the rabbit model. A 55% decrease in parvalbumin was seen in rabbit EDL muscle after 6 days of stimulation and levels were no longer detectable by 28 days as determined electrophoretically (Klug et al. 1983a). Similarly, 14 days of CLFS applied to rabbit TA muscle led to a 63% decrease in parvalbumin (Klug et al. 1983b). Using a more sensitive ELISA technique, Leberer and Pette (1986) found that parvalbumin protein content in rabbit EDL muscle was reduced by 50% and 95% after 10 and 50 days of stimulation, respectively.

In contrast, in rat EDL and TA muscles, Huber and Pette (1996) reported reductions in parvalbumin content only after 21 days of CLFS (10hrs/day) despite immediate downregulation of parvalbumin mRNA shortly after the onset of stimulation. They also concluded that in addition to transcription and translational modifications, protein degradation is important in regulating parvalbumin content during fibre type transitions. Similarly, in the present study, there were no observed decreases in parvalbumin content after 10 days of stimulation or 20 days of Cr feeding. When contrasted with previous work, which investigated chronic (13 weeks) Cr feeding and voluntary exercise (see Chapter 4), Cr feeding alone reduced parvalbumin content by 70%-83%. Thus, it appears that a substantial period of increased TCr is necessary before parvalbumin protein expression decreases. Under these conditions, it indicates

that parvalbumin is largely post-translationally regulated. This is consistent with the findings of Huber and Pette (1996), who reported that approximately a 21 day lag-time between full suppression of parvalbumin synthesis and the onset of parvalbumin decay.

5.4.3 *Calcium ATPase content*

The effects of CLFS on the expression of the proteins involved in excitation-contraction coupling have been extensively investigated over the past two decades (Hicks et al. 1997; Leberer et al. 1987; Matshshita and Pette 1992; Simoneau et al. 1989). Reports show that CLFS results in the downregulation of the fast SERCA1 content and the upregulation of the slow SERCA2 content at the mRNA level in rabbit muscles (Leberer et al. 1989) and at the protein level in rabbit and dog (Hämäläinen and Pette 1997; Briggs et al. 1989; Hu et al. 1995; Ohlendieck et al. 1991). The findings in the current study, however, show a lack of synchronicity between SERCA1 and SERCA2 protein expression: CLFS resulted in the reduction of the fast SERCA1 but did not alter slow SERCA2. An important novel finding of this study was that Cr feeding abolished the CLFS-induced decrease in the fast SERCA1 but not SERCA2.

A rapid, significant elevation in SERCA1 expression in the rat SOL at both the mRNA and protein levels after unloading (Schulte et al. 1993), contrasted with the more prolonged period (i.e. 28 days) before significant reductions in SERCA2 reveal a different time course in the remodelling process for these two Ca^{2+}ATPase isoforms. Similar changes in SERCA1 and SERCA2 protein content occurred after CLFS (Ohlendieck et al. 1999), denervation (Hämäläinen and Pette 2001) and muscle paralysis (Talmadge and Paalani 2007). Talmadge and Paalani (2007) showed an up-regulation of SERCA1 in rat SOL after 7 days of spinal cord transection while SERCA2 did not decrease until 15 days post-transection. Similarly, Ohlendieck et al. (1999) found that a rapid decline in SERCA1 within rabbit TA after 3 days of CLFS did not correspond to an elevation

of SERCA2: significant increases in SERCA2 were not seen until 30 days of stimulation. Thus, it appears that SERCA2 displays a slower time-course, and more importantly that SERCA1 is very responsive to changes in muscle activity. Clearly, the expression of the SERCA1 is more sensitive to changes in contractile activity than SERCA2, which may result from asynchronous turnover rates.

Alternatively, it is possible that the regulation of gene expression for these two proteins involves different signalling events, which remain to be delineated. In the rat, the fast SERCA1 isoform is encoded by the Atp2a1 gene located on chromosome 1 (Brandl et al. 1986) and is transciptionally regulated, while the SERCA2 isoform is encoded by Atp2a2 located on chromosome 12 (Shull et al. 2000) and appears that its regulation is more heavily influenced by post-transcriptional events (Schulte et al. 1993; Hu et al. 1995).

Calcium and/or ATP-dependent proteolysis could also account for the decrease in SERCA1 observed with CLFS alone, and the maintenance of high SERCA1 levels when CLFS was combined with Cr feeding. For example, CLFS elevates Ca^{2+} activated calpain proteolysis in skeletal muscle (Sultan et al. 2000), and calpain has been shown to regulate SERCA1 degradation in heart muscle (French et al. 2006). Additionally, increases in antioxidant capacity and lower oxidative stress associated with Cr feeding, (Lawler et al. 2002) probably also reduced SERCA1 protein degradation by ubiquitin proteolytic systems (Reid et al. 2001).

5.4.4 *Metabolic Profile*

CLFS induces increases in maximal mitochondrial enzyme activities, while reciprocally decreasing the activity of glycolytic enzymes (Hood and Pette 1989; Pette and Vrbova 1992, 1999; Pette and Simoneau 1988). The aerobic (CS and HADH) and glycolytic (GAPDH) reference enzyme activities measured in this

study are equilibrium enzymes that display zero kinetics in vivo, and thus their maximum activity is directly proportional to their enzyme content. The changes in metabolic enzyme reference measures are similar to previous rodent studies (Bamford et al. 2003; Skorjanc et al. 2001), and are consistent with normal mitochondrial genesis in response to CLFS (Hood and Pette 1989). As expected, CS and HADH were upregulated and GAPDH was downregulated by CLFS. A novel and interesting finding of the present study, was that GAPDH content remained elevated when Cr feeding was combined with CLFS. Although it is known that GAPDH expression is regulated by transcriptional, pre-translational, translational and post-translational events (Lowe et al. 2000), it is not known to what extent these events are influenced by the prevailing intracellular energy potential. In this regard it is interesting to note that Green et al. (1992) showed a strong correlation between CLFS-induced reductions in the intracellular energy potential and decreases in GAPDH activity within fast-twitch rodent muscles. Thus, it is possible that a higher intracellular energy potential in response to Cr feeding could have affected any one of the events leading to GAPDH expression. At the transcriptional level, there is evidence that this may involve Cr dependent decreases in myogenin (Hughes et al. 1999) or reduced calcineurin activity (Meissner et al. 2001).

In the present study, the results show that CLFS reduced CK content independent of acute Cr feeding. Similarly, Donoghue et al. (2005) reported that mitochondrial CK expression increased while the cytosolic isoform, which constitutes 90% of the total CK, decreased with CLFS in rabbit fast skeletal muscle. In contrast, Ingwall et al. (1975 and 1976) showed increased CK activity in primary cultures grown exposed to high levels of exogenous Cr. The in vitro resting model used in those studies typically produce muscle fibres with an immature embryonic phenotype (Wehrle et al. 1994), and thus limits direct comparison with the findings of the present study, which are based on mature, adult muscle fibre phenotype.

5.4.5 *Conclusions*

This is the first study to show that acute Cr loading during CLFS maintains a faster MHC-based phenotype as determined at the protein and mRNA level, while also promoting a more glycolytic metabolic profile. Furthermore, Cr loading abolished the CLFS-induced reduction in fast SERCA1.

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CHAPTER 6: General conclusions

6.1 SUMMARY

In the preceding chapters, fast-to-slow fibre type transitions were accelerated by increasing neuromuscular activity either voluntarily with wheel running (Chapters 3 and 4) or forcibly via CLFS (Chapter 5) in rat mixed fast twitch hindlimb muscles. The effects of Cr loading in combination with these two endurance regimens on muscle structure, function and metabolism were therefore investigated. A detailed morphological adaptation in response to changes in diet and activity were reported; patterns of MHC isoform expression were assessed at both the protein and mRNA level as determined by SDS-PAGE and real-time PCR, respectively. Pure and hybrid fibre types were also determined immunohistochemically to assess the cross-sectional areas of the various fibre types. Similarly, Ca^{2+} regulatory proteins (parvalbumin, fast SERCA1 and slow SERCA2) involved in the excitation-contraction cycle were studied in order to advance the understanding of how Cr loading might influence Ca^{2+} regulation. Isometric functional measures of force (twitch and tetanic force), speed (time-to-peak tension, half fall-time and half rise time) and fatigability reflected functional adaptations to Cr and activity treatments. Lastly, reference enzymes of oxidative (citrate synthase and 3-hydroxyacyl-CoA dehydrogenase) and glycolytic (glyceraldehyde phosphate dehydrogenase and phosphofructokinase) capacities, along with creatine kinase content were determined to demarcate the metabolic profile of muscles experiencing fast-to-slow fibre type transitions and how their contents are affected with Cr loading.

Unlike rabbits, rats exhibit all four MHC isoforms, and thus this animal model allows for the study of the full range of fibre type transitions. In addition, the rat was chosen as the experimental animal model because the induced fast-to-slow conversion in response to CLFS is a true fibre transition of uninjured fast adult fibres into slower fibres, whereas in the rabbit, fast degenerating fibres are

replaced or exchanged by slower fibres (Maier, Gambke and Pette, 1986). Thus, all MHC-based fibre type transitions from these studies are only due to true fibre transformation. The PL muscle was the primary focus of Chapters 3 and 4 because it possesses a mixed fast-twitch MHC-based phenotype and is heavily recruited during voluntary wheel running (Brannon et al. 1997 and Kariya et al. 2004). On the other hand, the TA muscle was the focus of the last study (Chapter 5) because it possesses a mixed fast-twitch MHC-based phenotype and is innervated by the common peroneal nerve, which can be easily targeted by CLFS application.

In the present studies, two distinct endurance training regimens were applied to induce fast-to-slow fibre type transitions. Nonetheless, two major differences exist between voluntary and forced elicited muscle contractions. During normal movement, motor units are activated asynchronously whereas electrical stimulation causes synchronous activity of all motor units. The hierarchical order of recruitment is cancelled by supramaximal electrical stimulation and the largest, normally least active units are stimulated simultaneously with other, usually more active motor units. Using CLFS as the experimental design includes other advantages: CLFS does not interfere with the innervation and natural activity delivered by nerve, muscle fibres can be activated by entirely novel impulse patterns, and it is unlikely that these patterns of stimulation will activate sensory nerves which may cause pain (Pette 2002).

The novel results from this dissertation can be summarized as follows:

- The isometric measures studied possess significant predictive value with regard to MHC isoform content; the left and right legs are interchangeable but display a considerable range (low to high) of reliability when only one hindlimb is studied (Chapter 2).

- As illustrated in Chapter 3, 13 weeks of Cr supplementation had differential effects on MHC isoform content and fatigability that depended on the level of contractile activity. Cr feeding combined with voluntary running exercise resulted in a faster MHC-based phenotype in the rat PL but the impact on associated isometric contractile properties was minimal.

- Chapter 4 further shows that Cr loading during run training maintains a faster muscle fibre phenotype and prevents increases in aerobic activity. Cr loading prevented decreases in fast SERCA1 but increased slow SERCA2 content while dramatically reducing parvalbumin protein expression, independent of run training.

- Lastly, acute Cr loading during CLFS maintained a faster MHC-based fibre phenotype as determined at the protein and mRNA level and promoted a more glycolytic metabolic profile. Cr feeding abolished the CLFS-induced reduction in the fast SERCA1 content but did not alter parvalbumin or slow SERCA2 content.

6.2 CREATINE INFLUENCES CELLULAR ENERGETIC STATUS

In 1992, Harris et al. first reported that oral Cr feeding could raise total intramuscular Cr content within human skeletal muscle. The following year, Greenhaff and colleagues (1993) observed increased muscle peak torque production during voluntary isokinetic contractions with oral Cr supplementation. Since then, over 100 studies have examined the effects of Cr supplementation on exercise performance. The focus of Cr research in exercise physiology has shifted from evaluating the impact on exercise performance to determining the mechanisms underlying the reported effects of oral Cr loading on muscle functional capacity.

Greenhaff et al. (1994) were the first to show that an increase in muscle Cr concentration due to Cr supplementation accelerates the rate of muscle PCr resynthesis during recovery from exercise, and could potentially improve fatigue resistance by enhancing the available pool of high-energy phosphates. Because of the role of Cr as a transport molecule of high-energy phosphate from sites of production to sites of utilization during increased levels of ATPase activity, it is believed that a higher ATP/ADP ratio can be maintained by an accumulation of PCr. However, McMillen et al. (2001) reported that Cr supplementation increased TCr in fast-twitch rat muscle without altering the resting ATP/ADP_{free} ratio as anticipated by the equilibrium nature of creatine kinase (CK).

Despite numerous Cr supplementation studies, it was still undetermined whether it is solely the increase in intramuscular TCr or the maintenance of a higher ATP/ADP_{free} ratio that is responsible for improved high energy phosphate shuttling. As discussed in Chapter 2, the resting ATP/ADP_{free} ratio within the MG and PL muscles remained unchanged by chronic Cr supplementation. Increased TCr levels therefore are accountable for the observed improved fatigue resistance after 10s of stimulation. Based on previous investigations (Bessman et al. 1987; Wallimann et al. 1992 and 1998), muscles which experience prolonged increases in TCr content have benefited from an enhanced rate and capacity for high-energy phosphate shuttling during activity. The studies of Ingwall et al. (1974 and 1976) showed that Cr selectively stimulated, by two-fold, the synthesis of actin, MHC and the cytosolic isoform of CK (MM-CK) in cultures of differentiating muscle cells. Interestingly, however, Cr did not alter the synthesis rates of soluble metabolic enzymes. Thus, it would seem that enhanced intracellular Cr combined with increased MM-CK expression has the potential to augment the efficiency and capacity for high-energy phosphate shuttling (Wallimann et al. 1998 and 1992), which could be expected to ameliorate local contraction-induced reductions in the IPP and influence fibre-type transitions, especially during exercise training.

As discussed in chapter 3, a persistent change in the IPP may act as an important physiological signal, contributing to fibre type transitions. To date, studies have only measured the ATP/ADP_{free} ratio at rest or shortly after activity. Therefore, it would be of great importance to investigate how this ratio is affected during contractile activity. A plausible method to study the ATP/ADP_{free} ratio during contractile activity would involve freeze-clamping the electrically stimulated rat hindlimb muscles as it is contracting, rapidly excising, and plunging it into liquid nitrogen for storage. A pilot study should be conducted in order to determine the optimal stimulation protocol to effectively decrease the IPP. Green et al. (1992) reported that electrical stimulation at 10Hz for 10hr effectively decreased the ATP/ADP_{free} along with PCr, Cr and TCr in the rabbit TA muscle. Based on his findings, a similar stimulation protocol could be used to lower the IPP of the rat skeletal muscles. The stimulation protocol would involve a continuous low frequency (10Hz) stimulation (0.380 ms single pulse duration, 6V) for 10hrs. Following the stimulation protocol the right and left muscles would be immediately freeze-clamped with metal clamps pre-cooled in liquid nitrogen (-196 °C), removed and stored in liquid nitrogen for assessment of intramuscular concentrations of TCr, PCr, Cr_{free}, free ADP and ATP via biochemical assays. Therefore, it would be possible to assess if increased TCr content in response to Cr loading is able to minimize the reduction of this ratio during contractility.

6.3 MYOSIN HEAVY CHAIN-BASED FIBRE TYPE ADAPTATIONS AFTER RUNNING AND CHRONIC LOW FREQUENCY STIMULATION COMBINED WITH CREATINE

Numerous studies have focused on the influence of decreased or increased neuromuscular activity on skeletal muscle fibre types. Adaptive responses to changes in contractile activity regarding MHC-based fibre type expression at both the protein and mRNA level in animal models are well documented (Pette 2002). In general, increasing neuromuscular activity results in fast-to-slow fibre type transitions, whereas reducing levels of activity leads to slow-to-fast fibre type transitions (Pette and Staron 2000). For example,

increasing neuromuscular activity (CLFS, functional overload, endurance training or strength resistance) results in increased proportions of either type I fibres and MHCI or type IIA fibres and MHCIIa while reducing neuromuscular activity (denervation, unloading, immobilization or spinal transection) results in increased proportions of type IID/X fibres and MHCIId/x or type IIB fibres and MHCIIb. In the present investigations, the models of increased neuromuscular activity (voluntary wheel running and CLFS) also resulted in fast-to-slow fibre type transitions as reflected by 6% and 12% increases in MHCIIa and MHCIId/x respectively, and a 19% decrease in MHCIIb in the PL muscles in response to running, and 2% and 15.2% increases in MHCI and MHCIIa, respectively and a 22% decrease in MHCIIb in the TA in response to CLFS. The transitions in MHC protein isoforms are preceded by similar changes at the mRNA level (Jaschinski et al. 1998). This occurred in the CLFS study (Chapter 5), where CLFS treatment greatly increased MHCIIa and decreased MHCIIb mRNA content, as reflected by lower and higher ΔCt values, respectively. Cr combined with these two models of increased neuromuscular activity attenuated these fast-to-slow fibre type transitions, and thus maintained a faster MHC-based phenotypic profile. When Cr was superimposed on running, the MHC-isoform transition occurred in the opposite direction; MHCIId/x → MHCIIb. When Cr loading was combined with CLFS, the increases in MHCI and MHCIIa and decreases in MHCIIb did not occur and MHCIId/x increased by 6%. Again, this attenuation was seen at the mRNA level where Cr combined with CLFS resulted in higher MHCIIb mRNA content compared to CLFS alone, as reflected by a lower ΔCt value (i.e. 6.1 vs. 9.1). Similarly, Cr loaded muscles maintained a higher MHCIId/x mRNA content, as reflected by a lower ΔCt value (i.e. 6.2 vs. 8.5).

The molecular mechanisms and signal pathways related to fibre type transitions occur at the transcriptional level, translational level and at the level of protein degradation. Changes in transcription and translation influence gene expression while proteolysis affects the balance between protein synthesis and degradation. Gene induction and repression are regulated by at least three

major signalling pathways. Ca^{2+} homeostasis plays a role in fast-to-slow fibre type transitions (Sreter et al. 1987) and involves the Ca^{2+}-calmodulin/calcineurin dependent pathway (Bigard et al. 2000; Chin et al. 1998; Naya et al. 2000). Transgenic mice expressing active calcineurin show upregulation of slow fibre type-specific genes (Naya et al. 2000). Treatment with cyclosporin A, a calcineurin inhibitor, induces reduced expression of MHCI in the slow SOL muscle (Bigard et al. 2000). Another pathway of signal transduction that includes Ca^{2+} concentrations and is involved in the control of muscle fibre phenotype is the Ca^{2+}-calmodulin-dependent protein kinase IV (CaMK-IV) (Wu et al. 2002). Mice expressing a constitutively active form of this protein, contained higher slow-twitch fibres and a more pronounced aerobic capacity. Ca^{2+} levels can also be altered by Ca^{2+}-sequestering proteins, which are influenced by ATP phosphorylation potential of the adenylate system (Conjard et al. 1998; Green et al. 1992). Reduced ATP phosphorylation potential impairs the function of Ca^{2+} ATPases, and thus affects Ca^{2+} sequestration by reducing Ca^{2+} uptake rates in the SR (Heilmann and Pette 1979). Taken together, Ca^{2+} initiates a signal chain that ultimately activates the transcription of genes encoding slow fibre type-specific proteins.

Lastly, ATP phosphorylation may influence gene expression by influencing 5'AMP-activated protein kinase (AMPK). This pathway increases mitochondrial enzyme activities, glucose transport (GLUT 4), hexokinase (HK) and CK (Holmes et al. 1999; Winder et al. 2000; Ponticos et al. 1998; Bamford et al. 2003; Putman et al. 2003). Depletion of PCr and ATP by β-guanidinopropionic acid (β-GPA) feeding in rats, activates AMPK and increases cytochrome c content, mitochondrial density and nuclear respiratory factor-1 (NRF-1), which encodes for mitochondrial transcription and replication machinery (Bergeron et al. 2001; Scarpulla 2002). CLFS onset decreases [ATP/ADP_{free}], resulting in the elevation of 5'AMP via the adenylate kinase reaction, which in turn appears to increase AMPK activity (Bamford et al. 2003; Putman et al. 2003; Putman et al. 2004).

Pathways involving AMPK thus affects levels of proteins involved in aerobic-oxidative and anaerobic metabolism.

It seems that these three major signalling pathways involved in slow gene promotion are dependent on the ATP phosphorylation potential and can ultimately be affected by increased intracellular PCr content via Cr supplementation (see Figure 6-1). However, whether the IPP alone is responsible for these MHC modifications is still unknown because changes within intracellular [Ca^{2+}] may still regulate gene expression. It is unknown whether the IPP works independently of the two already identified Ca^{2+}-dependent signalling cascades (Ca^{2+}-calmodulin/calcineurin dependent signal and Ca^{2+}/calmodulin-dependent protein kinase IV (CaMK-IV) pathways) involved in gene expression. Therefore, not only would it be interesting to assess the ATP/ADP_{free} ratio during activity, but furthermore determine whether its alterations are solely responsible for changes in phenotypic changes, independent of Ca^{2+} kinetics. The administration of calcineurin and CaMK-IV blockers in combination with β-GPA could allow for the direct assessment of the role of IPP as a potential regulator of gene expression. For example, cyclosporin A, a calcineurin blocker, and FN-93, a CaMK- IV blocker, administered to rodents via subcutaneous injections throughout a specified stimulation protocol should prevent the slow gene promoters from being the primary effectors, and thus reveal if decreasing IPP as a result of β-GPA feeding would result in a slower MHC-based phenotype. The purpose of such a study would determine if the IPP acts as a sole regulator of MHC isoform gene expression.

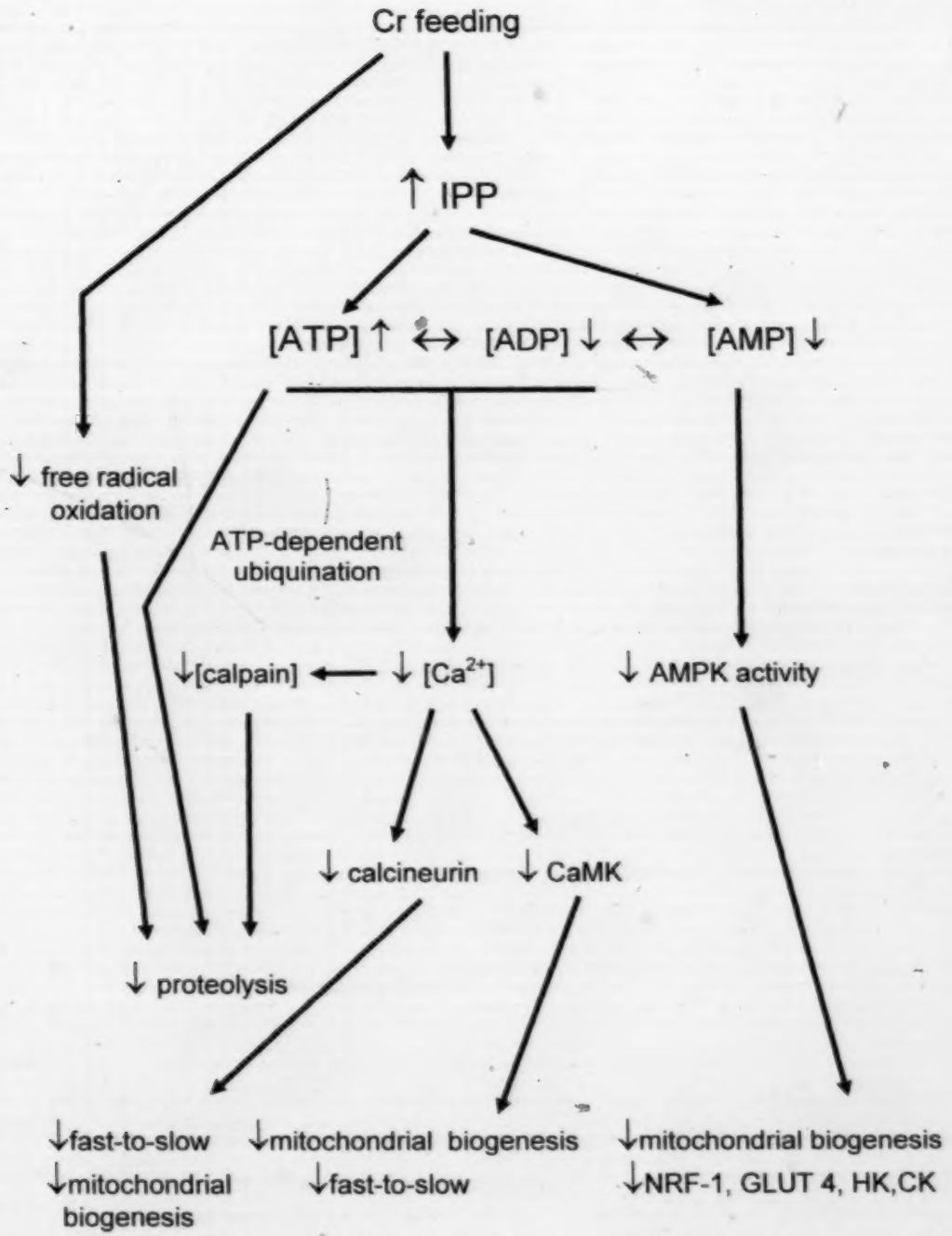
Calcium and/or ATP-dependent proteolytic pathways are also involved in fibre type transformations. Calpains are heavily involved in the myofibrillar remodelling process (Belcastro et al. 1998; Lecker et al. 1999). The elevation of μ-calpain activity in response to CLFS in rat TA muscle was associated with a corresponding increase in type IIA fibres, and was therefore suggested to play a role in the transformation process of the uninjured, mature fibres (Sultan et al.

2000). Calpain content and activity are controlled by numerous factors and among them are pH, ATP and Ca^{2+} levels (Twinning et al. 1994). Enhanced proteolysis by calpains is not restricted to fast-to-slow fibre type transitions in myofibrillar protein isoforms (Goodman 1987). They are also involved in enzyme metabolism (Pette and Vrbová 1992) and proteins involved in Ca^{2+} dynamics (Hicks et al. 1997; Ohlendieck et al. 1999). Thus, it follows that Cr supplementation could potentially affect Ca^{2+} and/or ATP-dependent proteolytic pathways in a manner that maintains higher levels of the faster MHC and SERCA isoforms and glycolytic enzymes.

Figure 6-1

Diagram of the potential influences of Cr feeding on muscle fibre type phenotype. Illustration of 3 major signalling pathways that could be involved in attenuating the activation of genes during fast-to-slow fibre type transitions and oxidative metabolism. Decreases in intracellular Ca^{2+} levels due to higher ATP and lower ADP levels would result in decreased calcineurin and CaMK activity, ultimately diminishing fast-to-slow fibre type transitions and mitochondrial biogenesis. Decreases in intracellular Ca^{2+} levels would also lower calpain content and decrease proteolytic activity. Decreases in AMPK activity due to lower AMP concentrations will reduce mitochondrial biogenesis and other factors (listed in the figure). Proteolysis is also influenced by ATP dependent-ubiquination and oxidative capacity, both which are affected by ATP levels and free radicals, respectively. Overall, Cr feeding leads to the maintenance of a faster fibre based phenotype while preventing increases in oxidative metabolism.

Figure 6-1


Cr feeding
↑ IPP
[ATP] ↑ ↔ [ADP] ↓ ↔ [AMP] ↓
↓ free radical oxidation
ATP-dependent ubiquination
↓[calpain]
↓ [Ca2+]
↓ AMPK activity
↓ calcineurin
↓ CaMK
↓ proteolysis
↓fast-to-slow
↓mitochondrial biogenesis
↓mitochondrial biogenesis
↓fast-to-slow
↓NRF-1, GLUT 4, HK,CK

6.4 DISCONNECTION BETWEEN MHC-ISOFORM PROTEIN EXPRESSION AND FUNCTIONAL PROPERTIES

The MHC molecule, the actin-based motor protein associated with muscle fibre contraction, plays a predominant role in specifying skeletal muscle properties (for reviews see Pette and Staron 1990, 2001 and Schiaffino and Reggiani 1996). Skeletal muscles of adult mammals contain 4-5 major MHC isoforms, one or two slow (MHCIβ, MHCIα) and three fast (MHCIIa, MHCIId/x MHCIIb). Essential and regulatory light chains both exist as "fast" and "slow" isoforms, forming different combinations of light and heavy chain isoforms that result in several functionally different isomyosins. Based on these different MHC complements, four fibre types have been delineated in adult skeletal muscles of mammals: fast types IIB, IID(X) and IIA and slow type I. There are pure fibre types, which express only a single MHC isoform, as well as hybrid fibres which are characterized by the coexpression of two or more MHC isoforms. Pure fibre types differ in their myofibrillar ATPase activity, tension cost and contractile properties; as shown by measures of maximum unloaded shortening velocity and stretch activation, type I are the slowest contracting fibres, within the fast fibre population, type IIB are the fastest, type IID(X) intermediate and type IIA are the slowest (Bottinelli et al. 1991). Therefore, differences in contractile properties have been assigned to fibre-type specific MHC isoforms (Bottinelli et al. 1994a and 1994b; Galler et al. 1994 and 1996). However, this assumption is weakened by the fact that the isoforms of myofibrillar proteins tend to coexpress and thus lead to overlapping MHC distributions. Tropomyosin-binding troponin subunits and myosin light chain isoform expression, which have been shown to play a role in contractile properties (Bottinelli et al. 1994), are also known to be coexpressed. Taken together, contractile properties are not merely under the influence of MHC isoforms but rather numerous factors.

As discussed in Chapter 2, contractile properties are not determined fully by MHC-isoform fibre type composition. Numerous factors influence force

measurements and speed of contraction and relaxation, along with measures of fatigability. Myosin ATPase activity is proportional to fibre contraction time (Bottinelli et al. 1994b) and measures of twitch and tetanic force. However, muscle architecture, including cross-sectional area of the fibres, muscle fibre length (sarcomeres in series) and muscle mass (sarcomeres in parallel) as well as pennation angle are known to impact isometric measures of force production. Tetanic force production may be influenced by ATP dependent Na^{2+} channels involved in the restoration of membrane potential between tetanic contractions; namely, increased number of transporters to restore the membrane potential could result in a higher tetanic force. Similarly, speed of contraction and relaxation are not merely dependent on myosin ATPase activity. TTP and ½ RT vary inversely with angle of pennation while ½ FT time varies proportionally with Ca^{2+} ATPase isoform expression, and parvalbumin content. Although not measured in these studies, myosin light chain content (ratio of MLC_{3f} to MLC_{2f}) (Brannon et al. 1997; Bottinelli 1991 and 1994) and troponin do influence the physiological measures of contractile speed.

6.5 DISCONNECT BETWEEN MHC AND CALCIUM REGULATORY PROTEINS

Various investigations report that the pattern of sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) isoform expression, like that of MHC, is under the control of contractile activity (Pette and Vrbová 1999). Hämäläinen and Pette (1997) have shown that CLFS increases slow MHC isoforms, slow isomyosins and SERCA2 in rabbit TA and EDL muscles. More importantly, a high correlation was found between relative concentrations of slow SERCA2 and slow MHCI isoforms ($r=0.97$) and fast SERCA1 was correlated with fast myosin isoforms. Thus, suggesting that the transitions in myosin and Ca^{2+} ATPase isoforms occur in a coordinated manner and that regulation of expression for these two different proteins may be under a common control. Similarly, a very high degree of correlation in the proportion of fibres expressing slow SERCA2 and MyHCI was found by Talmadge et al. (1996). Both studies, however, assessed these

adaptations at only one long-term time point and do not determine if mismatching of SERCA and MHC occur during the transformations (72d in Hämäläinen and Pette study and 3 months in Talmadge et al. study)

Additionally, Hicks et al. 1997 reported alterations in excitability, Ca^{2+} handling, and excitation-contraction coupling prior to changes in myofibrillar protein isoforms, and may thus be responsible for early functional alterations seen with CLFS in the rabbit TA. However, changes in SERCA1 in response to CLFS were not seen until >10 days of stimulation, suggesting that the Ca^{2+} ATPase activity, not the expression, of the SERCAs is the underlying molecular mechanism preceding changes in MHC-based phenotypes and perhaps causing changes in relaxation times. As mentioned in Chapter 5, this may explain why there was a mismatch between SERCA and parvalbumin content and the observed ½ fall time in the TA muscles. Huber and Pette (1996) showed that CLFS suppressed parvalbumin expression in rat EDL and TA muscles. The time course of disappearance with CLFS onset and the reappearance and rapid increase in parvalbumin mRNA soon after cessation of CLFS parallels that of MHCIIb mRNA, thus supporting the notion of coordinated expression. Mismatches between SERCA and MHC expression have been reported in muscle paralysis (Talmadge et al. 2007) and CLFS (Mabuchi et al. 1990) studies where a high proportion of fibres expressing slow MHC retained fast SERCA1 protein expression. A speculative explanation for this phenomenon is that SERCA1 may be a very sensitive marker and precedes changes in MHC-isoform transitions.

6.6 CLINICAL RELEVANCE

Although the majority of studies on Cr have been on exercise performance in healthy humans, recent evidence suggests that Cr may be helpful in treating diseases characterized by muscle atrophy or muscle fatigue secondary to impaired energy production. Cr is involved in energy production and acts as a

shuttle of ATP from the inner mitochondria to the cytosol, and thus may be useful in diseases of mitochondria where energy production is altered. Cr supplementation has been a neuroprotective agent in diseases in which there is mitochondrial dysfunction such as Parkinson's (Matthews et al. 1999), Huntington's (Matthews et al. 1998; Ferrante et al. 2000) and amyotrophic lateral sclerosis (Klivenyi et al. 1999). Tarnoplosky and Martin (1999) investigated Cr supplementation in various neuromuscular diseases including mitochondrial cytopathies, neuropathic disorders, dystrophies and myopathies. They found increases in high-intensity strength measures and lean body mass following Cr supplementation of 10g/day for 5 days with 5g/day for 5-7days of maintenance. Tarnopolsky et al. (1997) also found Cr (2 X 5g/day for 2 weeks with 2 x 2g/day for 1 week maintenance) to be beneficial for patients with mitochondrial cytopathies. It was concluded that Cr increased strength and high-intensity anaerobic and aerobic activities with no effect on low intensity aerobic activity. Similarly, Rodriguez et al. (2007) found that treatment with a combined therapy of Cr, coenzyme Q_{10} and α-lipoic acid resulted in lower resting lactate levels, prevention of a reduction in peak ankle dorsi-flexion strength, and a lowering of oxidative stress in patients with various mitochondrial cytopathies and disorders.

Felber et al. (2000) reported increases in strength with Cr supplementation (10 g/day for adults and 5 g/day for children for 8 weeks) in humans with various muscular dystrophies. Pulido et al. (1998) reported that Cr treatment of cell cultures isolated from *mdx* mice, a murine model of Duchenne muscular dystrophy, decreased sarcolemmal leakage of Ca^{2+}, thus reflecting an increase in mechanical stability. The increases in Cr content could contribute to the attenuation of the deterioration of intracellular energy homeostasis, and may thus be one of the factors preventing the aggravation of muscle weakness and degeneration Overall, these studies suggest that Cr supplementation improves high-intensity exercise performance and absolute strength in a variety of patients with neuromuscular disorders.

Cr intake will thus enhance muscle fibre regeneration and improve muscle performance (strength and power output), which declines in these neuromuscular diseases. Cr feeding may also play a role in the prophylaxis or treatment of steroid-induced myopathy (Menezes et al. 2007). Cr feeding attenuated dexamethasone-induced gastrocnemius and diaphragm muscle weight losses and the atrophy of gastrocnemius type IIb fibres in rats. In addition, Cr has exerted protective effects in a variety of pathologies where oxidative stress plays an etiologic role. Cr displayed direct antioxidant activity in the human and murine cell lines; cytoprotection was associated with elevation of the intracellular fraction of Cr via a mechanism depending on direct scavenging of reactive oxygen (in particular hydroxyl radical) and nitrogen species (Sestili et al. 2005). Lastly, Berneburg et al. (2005) observed that increase of the energy precursor Cr protects from aging-associated mutations of mitochondrial DNA, which play a role in neurodegeneration. It was hypothesized that Cr indirectly protects against mitochondrial DNA mutations by normalizing the cell's energy status, and consequently reducing the requirement to upregulate a deleterious respiratory chain that regenerates more reactive oxygen species (ROS). Further experiments are needed to clarify the interaction between ROS, mitochondrial mutagenesis, and energy metabolism, but the anti-oxidant effect of Cr may serve as a tool to further investigate the underlying mechanisms of processes such as aging and carcinogenesis.

6.7 REFERENCES FOR CHAPTER 6

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